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SECTION MEETINGS

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14159

Stability of Acid-Base Equilibrium of Blood in Animals Falling in Different Age Periods.*

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The following data have been obtained by a review of experiments conducted some years ago. At such periods of investigation changes in the acid-base equilibrium of the blood were ascertained by determining the alkali reserve of the blood plasma by the method of Marriott.[4] This technic was in use during the period of the development of the more accurate method of Van Slyke, which statement serves as an explanation for not having employed in these experiments this more dependable method for ascertaining changes in this fundamental equilibrium of the animal organism.

The following observations have been made on a series of 89 dogs which on the basis of their ages may be divided into 3 age groups. Group I, is represented by 21 puppies not over 6 months in age. Group II, by 33 adult dogs that varied in age from 1 to 6 years, while Group III, is composed of 35 old or senile animals varying in age from 9 years to 19 years and two months.

Control studies of the animals in the respective groups have shown all of the animals with the exception of 6 dogs falling in the senile group (Group III) to be free from evidence of renal injury. The urine of 6 animals referred to contained a constant trace of albumin, broad hyaline casts and occasional red cells. The elimination of phenolsulphonephthalein in an hour period by these animals was below the normal value for this evidence of renal function, giving readings as low as 28

* Observation of a similar order have appeared from this laboratory in past years.[1,2,3]

[1] MacNider, W. deB., *Science*, 1921, **53**, 141.

[2] MacNider, W. deB., *J. Exp. Med.*, 1926, **43**, 53.

[3] MacNider, W. deB., *J. Exp. Med.*, 1917, **36**, 1.

[4] Marriott, W. McKim, *Arch. Int. Med.*, 1916, **17**, 840.

and 22%. There was no retention of non-protein nitrogen. The determinations of the reserve alkali of the blood for the animals of all 3 groups including those dogs in Group III which gave evidence of renal impairment were normal and varied between 8.05 and 8.15. Such determinations were made in duplicate on 6 different days.

The experimental procedure employed to ascertain the stability of the acid-base equilibrium of the animals has consisted in giving the dogs subcutaneously 0.25 cc of a 2% solution of morphine sulfate and following the initial period of excitement when the animals gave evidence of a variable degree of narcosis, they were then given, by the open method of administration, chloroform for a period of 45 minutes. The following results have been obtained in the animals of the respective groups. In Group I, represented by the puppies, the reserve alkali has shown a reduction in all of the animals and in 6 of the 21 dogs a reduction from the normal of 8.15 to 7.75. In this group regardless of the degree of depletion in the alkali reserve a return to a normal equilibrium has been re-established within 1 hour and 20 minutes following the discontinuance of the anesthetic. In Group II, represented by 33 adult animals, there occurred no reduction in the alkali reserve in 11 members of the group. In the remaining 22 dogs in which a depletion was effected the determinations did not depart from the normal readings of from 8.1 or 8.5 to a reading lower than 7.85. In this adult group (Group II) the restoration of the alkali reserve to the normal was slower than in the group of puppies (Group I). With 3 exceptions a readjustment was only established within 1 hour and 50 minutes. In Group III representing the older and senile animals with the exception of the 6 animals that gave evidence of renal impairment, the use of the anesthetic body induced a rapid reduction in the alkali reserve to a point indicating a greater degree of depletion, 7.85 to 7.65, than had occurred in the adult animals of Group II. In this respect the instability of the acid-base equilibrium of the blood resembles the type of reaction obtained in the puppies of Group I. However, in the ageing and senile animals of Group III there is a marked delay in the restoration of this balance. With 4 exceptions a time limit of 2 hours and 40 minutes was necessary for the equilibrium to be re-established. In this respect, the senile animals of Group III differ from the puppies of Group I. Finally, falling in Group III, as has been indicated, 6 dogs gave evidence of renal impairment which was shown by a reduction in the elimination of phenolsulphonephthalein and by certain abnormal changes in the urine. The use of the anesthetic body in these animals for a period of 45 minutes has failed to induce a reduction in the reserve alkali of the blood below that figure which the respective animals had established as their normal. This has varied from 8.0 to 7.9. It would appear that in such animals even though the normal alkali reserve readings were at a low normal, 8.0, or at a point below this, 7.9, they had become through some mechanism established as stabilized values.

Conclusions. 1. Adult dogs varying in age from 1 to 6 years give greater evidence of stabilization in the alkali reserve of the blood than do young dogs, puppies, with an age limit of 6 months or older and senile animals varying in age from 9 years to 19 years and 2 months. 2. In certain instances dogs with evidence of renal injury may stabilize against strain the alkali reserve of the blood at a point below the normal determination for such a value.

14160

Morphologic Effects of Poliomyelitis Virus upon Motor End Plates in the Monkey.*

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No report has been found of observations on the early morphologic disappearance of motor end plates and early appearance of ephemeral inclusion bodies in striped muscle coincident with or following shortly after the onset of paralysis in acute poliomyelitis in the monkey. The cellular and vascular reactions in the central, and in parts of the peripheral, nervous systems during the acute stages have been exhaustively studied.[1-11]

Methods. Motor end plates in the triceps, biceps brachii, and quadriceps femoris were studied in 8 monkeys, and in addition to these the gastrocnemius and tibialis anterior muscles in 7 monkeys, inoculated with poliomyelitis virus. Eight monkeys (*Macacus rhesus*) were chloroformed from 8 to 20 days after inoculation intracerebrally with 0.5 cc of 5% virulent cord-emulsion (Armstrong-Lansing strain of poliomyelitis virus).† Parts of each muscle were fixed and stained by different histologic technics. The best one for demonstrating the pathologic changes in the anatomic continuity of axis-cylinders, motor end plates, and striped muscle was a modified gold method,[12] followed by careful teasing of small pieces (2 x 5 mm) of the impregnated muscle. This report was limited to the findings obtained by the gold method. The brief protocol of monkey No. 490 (Kramer's series), from which photographs (Fig. 2 to 6) were made of differential nerve degeneration follows: intracerebral inoculation 2/14/43; rise of temperature 2/25/43; paralysis of fore limbs 2/27/43; paralysis of hind limbs, complete quadriplegia, chloroformed 3/3/43. Thirty selected muscles from both fore and hind limbs in 2 normal monkeys were prepared by the same gold method applied to the paralyzed muscles and used as controls. More than 2000 teased preparations of the innervation of different muscles were used in this study.

Results. The normal motor end plates (Fig. 1) varied from 15 to 30 μ, measured

* Sponsored by the National Foundation for Infantile Paralysis, Inc.

1 Landsteiner, K., and Levaditi, C., *C. R. Soc. Biol.*, 1909, **67**, 592, 787.

2 Flexner, S., and Lewis, P. A., *J. Exp. Med.*, 1910, **6**, 330.

3 Taylor, H. D., *J. Exp. Med.*, 1919, **29**, 97.

4 Penfield, W., *Am. J. Path.*, 1928, **4**, 153.

5 Fairbrother, R. W., and Hurst, E. W., *J. Path. and Bact.*, 1930, **33**, 17.

6 Hurst, E. W., *J. Path. and Bact.*, 1929, **32**, 457; *ibid.*, 1931, **34**, 337.

7 Corell, W. P., PROC. SOC. EXP. BIOL. AND MED., 1930, **27**, 927.

8 Toomey, J. A., and Takacs, W. S., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 319.

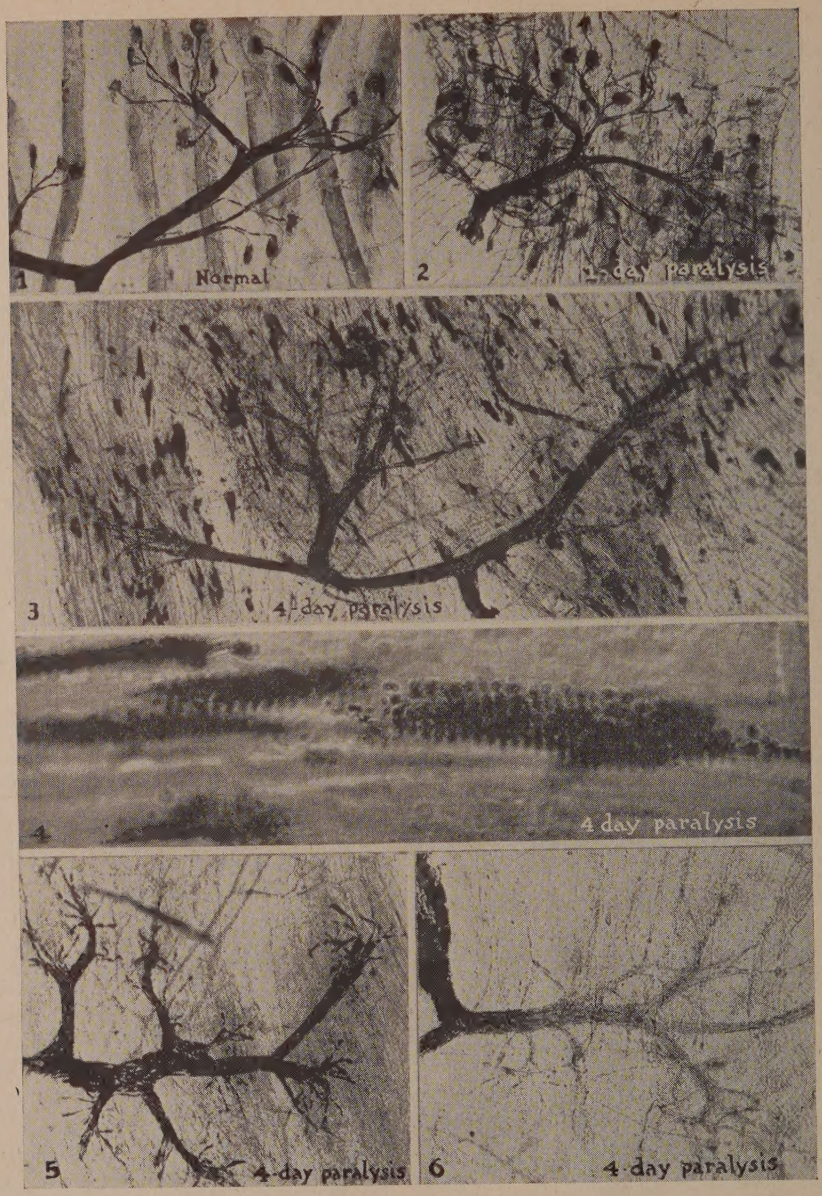
9 Sabin, A. B., and Olitsky, P. K., *J. A. M. A.*, 1937, **108**, 21.

10 Goodpasture, E. W., *Infantile Paralysis Symposium*, Vanderbilt University, National Foundation for Infantile Paralysis, Inc., 1941, 85.

11 Howe, H. A., and Bodian, D., *Neural Mechanisms in Poliomyelitis*, The Commonwealth Fund, 1942, 4 and 78.

† We are deeply indebted to Dr. S. D. Kramer, Michigan Department of Health, Lansing, for muscles from 8 monkeys; to Dr. F. J. Moore, Los Angeles County Hospital, and Dr. J. F. Kessel, University of Southern California, for 5 inoculated monkeys; to Dr. P. F. Clark and Dr. A. F. Rasmussen, University of Wisconsin, Madison, for muscles from 1 inoculated and 4 normal monkeys; to Dr. H. E. Pearson, School of Public Health, University of Michigan, Ann Arbor, for muscles from 1 monkey; for photographic and technical assistance to L. C. Massopust, E. Haushalter, J. Keyes and J. Schmitz, Marquette University; and to Dr. John L. Lavan, Director of Research, The National Foundation for Infantile Paralysis, Inc., for obtaining the cooperation of many laboratories in this study.

12 Carey, E. J., *Am. J. Path.*, 1942, **18**, 237.



Photomicrographs, normal innervation, quadriceps muscle in the monkey, Fig. 1, × 70; innervation, quadriceps, 1 day after paralysis, Fig. 2, × 70; biceps brachii 4 days after paralysis: degenerating innervation has multiple masses of inclusion bodies in muscle fibers, Fig. 3, × 70, and Fig. 4, × 650; triceps 4 days after paralysis: total absence of motor end plates, Figs. 5 and 6, × 70.

in the longitudinal axis of the quadriceps muscle fiber. The dark and light muscle fibers were evident in the normal Fig. 1, but not in the paralyzed, Fig. 2, quadriceps muscle. Some end plates on the first day of paralysis were retracted in a ball-like mass, Fig. 2, with shortened axis-cylinders and intense affinity for gold; others were hypertrophied and granular with loss of definition; many plates were very small, granular, and had weak affinity for gold. About 20% of the motor plates were absent in the paralyzed muscle on the first day; whereas within 2 to 4 days about 50% of the end plates had disappeared. On the 4th day, many retracted axis-cylinders were completely denuded of end plates, Fig. 5, and had sharp or budlike ends in the triceps muscle. The rates of nerve degeneration were unequal in this muscle. Some trees of axis-cylinders had advanced granular degeneration and weak affinity for gold at their terminals, Fig. 6, near the zone of the absent end plates. Proximad, the same axis-cylinders had a normal morphology and strong affinity for gold. The direction of degeneration appeared to be centripetal from the end plates in many fields. The intramuscular blood vessels were congested, cuffed, and had more granules, with strong affinity for gold, in the lumen than the normal. Masses of inclusion bodies, Fig. 3 and 4, which varied in length from 10 to 85 μ, were found within many muscle fibers in 4 of the 8 monkeys (Kramer's series) killed from 1 to 4 days after the onset of paralysis. These auriphilous inclusion masses were composed of granules 0.1 to 2 μ and spheroid bodies 2 to 4.5 μ in diameter. Each isolated body was frequently surrounded by a halo. In many instances, the finer constituents were arranged in cross striations, Fig. 4, which were more intensely stained with gold than those of the muscle fibers. Care was taken to differentiate histologically these masses of inclusion bodies from sporidiosis. In 15,179 muscle fibers of the right biceps, monkey No. 490, these inclusion masses were found in 12,683 fibers near the zone of the disintegrating innervation. They have not been found in 30 muscles of 2 normal control monkeys nor in those after one week of paralysis. These ephemeral inclusion masses at the degenerating myoneural junction appeared to be characteristic, as far as this limited evidence goes, of certain unknown chemical reactions that occur at certain times during the early stages of experimental poliomyelitis within and near the degenerating innervation of muscles in some monkeys. This study will be continued on a large series of mice.

Summary. Coincident with and shortly following paralysis by poliomyelitis virus the following morphologic changes occur in the motor end plates: 1, disappearance of many end plates resulting in denervation at the myoneural junction; 2, ephemeral appearance of masses of inclusion bodies, some of which are cross striated, within and near the degenerating motor end plates; 3, differential rates of motor nerve degeneration; 4, degeneration beginning in the motor end plates and proceeding in a centripetal direction in the axis-cylinders of many motor nerves.

14161 P

Effect of Low Atmospheric Pressures on Reproductive System of the Male Rat.

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Adult male rats (200-280 g) were exposed to pressures of 280-250 mm Hg. (25-28,000 ft) 6 hours daily for 14 to 18 days. The mean testicular, seminal vesicle and ventral prostate gland weights ± the Standard Error are shown in Table I. Body weight losses in the experimental animals averaged only 8.7%.

Examination of sections of testes from low pressure-exposed rats (Group A) showed marked degeneration of the spermatogenic cells. Polychromasia and coalescence of cytoplasm, pyknosis of nuclei, and chromatolysis were observed in these reproductive elements. The tubular lumina were devoid of sperm. The interstitial tissue of the testis and epithelium of the seminal vesicle and prostate gland, however, appeared only slightly atrophied. Pituitary glands of the low pressure-subjected animals displayed increases in the numbers of basophiles. Some of these were enlarged and showed signs of degranulation. Others resembled the vacuolated cells occurring in pituitary glands after castration.

The gonadotropic hormone contents of the pituitary glands from normal and low pressure-subjected rats were determined in 48 immature female rats employing the method of Reece and Weatherly.[1] Three assay experiments were performed using pituitary glands from 3 different sets of animals. In each case, the gonadotropic hormone potency was found to be significantly greater in the glands obtained from the low pressure-exposed rats. The mean ovarian weight in the test animals injected with pituitaries from low pressure-exposed animals was 88.3 ± 6.5 mg as against a mean ovarian weight of 62.8 ± 3.9 mg in rats receiving normal pituitary glands.

In another experiment, 8 rats were subjected to the same low pressure treatment but, in addition, were injected intrascrotally twice daily with 20 I.U. pregnancy urine hormone and 10 R.U. pregnant mare serum extract for 3 days preceding and during the entire period of exposure. As seen from Table I (Group B) the hormone treatment succeeded in elevating the testicular weights but these did not attain normal values. The injections, however, resulted in a marked increase in the weights of the reproductive accessories. Histological studies of the testes from these hormone-treated rats showed no repair of the gametogenic tissue but considerable hypertrophy and hyperplasia of the interstitial cells. The failure of the hormone material to restore spermatogenesis in the low pressure-exposed animals was not due to inadequate quantities of follicle-stimulating hor-

TABLE I.

Group	Treatment	No. of animals	Testes (mg)	Seminal vesicles (mg)	Ventral prostate (mg)
A	Low Pressures	47	1718.5 ±52.9	204.5 ±8.8	201.7 ±11.0
B	Low Pressures + Gonadotropin	8	2110.3 ±115.5	336.4 ±24.6	413.3 ±25.4
C	Controls	44	2443.1 ±46.3	264.3 ±10.8	318.0 ±12.4

1 Reece, R. P., and Weatherly, E. J., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 218.

mone in the injected extracts. Administration of the same hormonal treatment to 3 hypophysectomized non-exposed rats resulted in complete repair of the germinal tissue along with marked development of the interstitial cells of the testis.

It is concluded that the degenerative changes described in the male gonad are due to a direct inhibitory action of the low atmospheric pressures on the germinal tissue of the testis. The histological and gonadotropic hormone content changes in the anterior lobe of the pituitary, for the period of low pressure exposure employed, probably represent a reflection of this inhibition.

14162

Local Anesthetic Activity of a Number of New Monohydrochlorides of Dialkylaminomethyl Phenyl *p*-Aminobenzoates.*

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The present studies are concerned with an appraisal of the local anesthetic activity of a number of new phenyl *p*-aminobenzoates (Table I) synthesized by Dr. R. L. Shriner† and associates in the Department of Organic Chemistry, University of Illinois. The technic described by Rose[1] was used to detect dermal anesthesia. Aqueous solutions of the compounds were injected in 0.1 cc volumes in guinea pigs shaved free of hair and the presence or absence of anesthesia determined by faradic stimulation of each area injected. Solutions prepared just prior to testing were used because precipitation of all derivatives is apparent shortly after preparation. As shown in Table I the new aminobenzoates produced insensitiveness to electrical stimulation for 79-99 minutes in 1.0% and 31-79 minutes in 0.5% concentration. In both 1.0% and 0.5% solution all compounds produced hyperemia followed in most cases by necrosis at the site of injection.

In order to assess topical activity, freshly prepared aqueous solutions of derivatives (I-IX) were instilled into, and allowed to remain in the conjunctival sac of rabbits for 2 minutes. Anesthetic activity was determined by touching the cornea with the rounded end of a fine glass rod. Absence of the winking reflex was taken as evidence of anesthesia. As shown in Table I, compounds I, II, and III in 1.0% solution were inactive. While definite anesthesia was not observed, a sluggish reflex occasionally was noted for 5-10 minutes after application of these substances. In 1.0% concentrations IV, V, VI, VII, VIII and IX exhibited anesthetic activity for average periods of 21-73 minutes (Table I). The depth of anesthesia after V, VIII and IX was greater than that produced by IV, VI and VII. In the present studies, local application of 1.0% cocaine hydrochloride caused anesthesia for an average duration of 18 minutes. The corneal reflex was abolished for an average of 50 minutes after compound IX in 0.5% solution but in this concentration all of the other derivatives were ineffective.

Since IX appeared to be the most interesting of the new compounds, its acute toxicity after subcutaneous injection in mice was compared with that of cocaine hydrochloride. The MLD of IX was found to be approximately 300 mg/kg and that of cocaine between 100-150 mg/kg (Table II).

Summary. Intradermally in guinea pigs the hydrochlorides of 9 new dialkylaminomethyl phenyl *p*-aminobenzoates were found

* This investigation was supported in part by the D. J. McCarthy Foundation.

† Samples of these compounds were made available for the present experiments by Dr. R. L. Shriner, Department of Organic Chemistry, University of Indiana.

1 Rose, C. L., *J. Lab. Clin. Med.*, 1929, **15**, 128.

TABLE I.
Duration of Local Anesthesia.

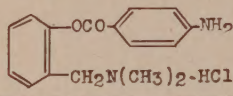
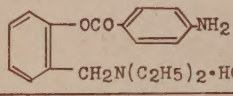
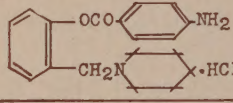
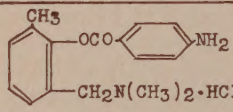
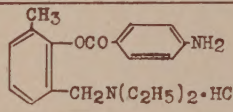
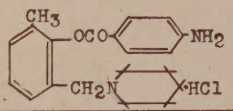
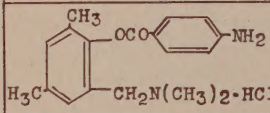
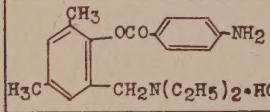
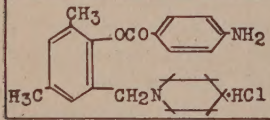
Formula	Code No.	Decomposition. °C.	Duration of Anesthesia in Guinea-pigs (Intracutaneous injection) Minutes		Duration of Corneal Anesthesia in Rabbits Minutes	
			1.0%	0.5%	1.0%	0.5%
	I	125-135°	88	69	0	-
	II	120-128°	94	62	0	-
	III	118-130°	79	31	0	-
	IV	185-190°	93	62	28	0
	V	180-185°	87	59	30	0
	VI	168-176°	92	75	21	0
	VII	199-203°	88	32	34	0
	VIII	143-149°	99	79	39	0
	IX	193-195°	93	66	73	50

TABLE II.
Subcutaneous Toxicity in Mice.

Compound	Dose, mg/kg	Mice died/Mice used
IX	250	2/13
	300	8/10
	350	9/10
	400	10/10
	500	10/10
Cocaine	75	2/10
	100	4/10
	150	9/10

to produce local anesthesia in 1.0% and 0.5% solution but all caused tissue damage at the site of injection. In 1.0% solution certain of these derivatives abolished the corneal reflex after instillation in rabbit eyes. The hydrochloride of (2-piperidinomethyl-) 4,6-dimethylphenyl *p*-aminobenzoate (IX) was less toxic subcutaneously in mice and exhibited greater topical activity in rabbits than cocaine hydrochloride but practical application of the anesthetic action of IX or any of the new compounds is complicated by their instability in aqueous solution.

I wish to express my thanks and appreciation to Dr. A. E. Livingston for helpful suggestions and encouragement.

14163

Antihemorrhagic Vitamin Effect of Honey.*

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While working on the role of honey in the prevention and cure of nutritional anemia in rats, one of the authors was impressed by the fact that the coagulability of blood in the rats receiving a honey supplement was so high that it was sometimes extremely difficult to draw samples for the hemoglobin determinations. The natural antihemorrhagic vitamins K are fat soluble substances. However, Warner and Flynn[1] have shown that there are water soluble substances which show antihemorrhagic activity. Therefore, it was considered advisable to test honey for the presence of factors influencing the coagulability of blood.

The method followed was a slight modification of the vitamin K assay devised by Almquist[2] using chicks. Herring meal was used in the basal diet instead of sardine meal and the temperature of the thermostat in which coagulability was determined was 37°C instead of 38°-39°C. In this procedure the only variable is the prothrombin level of the blood. Under these conditions the coagulability is referred to as "prothrombin time." Almquist[2] has found that a direct proportionality exists between 10 × (reciprocal of the prothrombin time, in seconds) and the log (10 × vitamin K intake).

Three series of experiments were performed, shown in Tables I, II and III. These were carried out at different times using 3 different but fresh preparations of thromboplastin for determination of prothrombin time. Each series required a different set of normal and negative controls.

In the first series, shown in Table I, mixed sweet clover and white clover honey, alfalfa and buckwheat honey were tested. The first 2 were mixed with the vitamin deficient ration in the ratio of 1:4 by weight; the buckwheat honey was added to the ration in the ratio of 1:3. The supplements were offered for 4 days to the vitamin K deficient chicks after the depletion period. The control chicks were given a solution of 2-methyl-1,4 naphtho-

* Paper No. 1995, Scientific Journal Series, Minnesota Agricultural Experiment Station, St. Paul.

1 Warner, E. D., and Flynn, J. E., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 607.

2 Almquist, H. J., *J. Assn. Off. Agr. Chem.*, 1941, **24**, 405.

TABLE I.

Antihemorrhagic Vitamin Effect of Honey.

Diet	Total supplement (4 days)	No. of chicks	Prothrombin time: Mean and standard error, sec.	Prothrombin time: Coeff. of variability, %
Normal diet	—	5	44 ± 1.0	4.7
Basal + 0.5 μg stand. vit. K in ethyl laurate (once daily)	2 μg	7	237 ± 4.6	4.7
Basal + 2.0 μg stand. vit. K in ethyl laurate (once daily)	8 "	8	59 ± 1.9	8.3
Basal containing 20% mixed honey	8 g	15	210 ± 48.0	85.8
Basal containing 25% buckwheat honey	10 "	12	173 ± 3.3	8.4
Basal containing 20% alfalfa honey	8 "	14	228 ± 3.9	6.3
Basal only	—	6	>1 hr	—

TABLE II.

Effect on Prothrombin Time of Administering Standard Vitamin K in Water vs. in Ethyl Laurate.

Diet	Total supplement (4 days)	No. of chicks	Prothrombin time: Mean and standard error, sec.	Prothrombin time: Coeff. of variability, %
Normal diet	—	5	49 ± 1.3	5.3
Basal + 2 ml aqueous susp. vitamin K nearly equal to 3 μg (once daily)	12 μg	20	157 ± 5.2	16.1
Basal + 3 μg stand. vit. K in ethyl laurate (once daily)	12 "	10	51 ± 0.5	2.8
Basal only	—	7	>1 hr	—

TABLE III.

Lack of Vitamin K Activity of Alfalfa Hay and Honey When Administered in Water Suspension and Restoration of Normal Effect by Giving Ethyl Laurate.

Diet	Total supplement (4 days)	No. of chicks	Prothrombin time: Mean and standard error, sec.	Prothrombin time: Coeff. of variability, %
Normal diet	—	7	40 ± 1.0	6.4
Basal + 0.5 μg stand. vit. K in ethyl laurate (once daily)	2 μg	5	176 + 3.8	4.4
Basal + 2 μg stand. vit. K in ethyl laurate (once daily)	8	6	48 ± 1.3	5.9
Basal + 2 ml 10% susp. alfalfa hay (once daily)	8	7	dead of hemorrhage	
Basal + 2 ml 10% susp. alfalfa hay + 2 drops Et. laurate (once daily)	8	9	126 ± 4.5	10.1
Basal + 2 ml 50% sol. alf. honey (3 times daily)	12	8	1686 ± 396	61.7
Basal + 2 ml 50% sol. mixed honey (3 times daily)	12	5	972 ± 108	21.8
Basal + 2 ml 50% sol. buckwheat honey (3 times daily)	12	6	912 ± 66	17.1
Basal only	—	6	>1 hr	—

quinone in ethyl laurate orally by means of pipette, and were not allowed any access to the food and water for half an hour after dosing.

Assuming the proportionality between prothrombin time and vitamin K intake found by Almquist, it may be calculated from graphs constructed from our results that the honeys tested when mixed with a vitamin K deficient ration showed a definite antihemorrhagic activity equivalent to approximately 0.25 μg of 2-methyl-1,4 naphthoquinone per gram of honey. However, when an aqueous solution of honey was administered directly to vitamin K depleted chicks, the prothrombin time was considerably greater. The actual dilution employed was not recorded and no attempt made to determine actual clotting time. This result was so surprising that it seemed to warrant further study.

It is known that the bile favors the utilization of vitamin K.[3,4] The suggestion was therefore advanced that when an aqueous solution of honey is administered to chicks normal bile secretion does not occur, thus preventing an adequate absorption of the antihemorrhagic vitamin. To test this hypothesis, 2-methyl-1,4 naphthoquinone was administered by pipette to vitamin K deficient chicks in aqueous suspension with and without the addition of ethyl laurate. The results presented in Table II show that a direct feeding of vitamin K in a water suspension increased the prothrombin time about 3 times over that of the normal controls or when the vitamin was administered in ethyl laurate.

Since alfalfa hay is known to be a good source of vitamin K a further study of the type of dosing procedure was made by comparing the effect of administering this material in aqueous suspension with and without ethyl laurate with the effect of the standard vitamin K with ethyl laurate. A further study was also made at this time of administering a solution of 3 different types of honey, concentrated, *i.e.*, 50%, solutions being used. The results given in Table III show that the chicks fed alfalfa hay in aqueous suspension died of hemorrhage while an addition of only 2 drops of ethyl laurate to this suspension brought the prothrombin time to about 3 times the normal. Graphs constructed from the results of the tests with the standard vitamin in series A and C, Tables I and III, correlating the reciprocal of the prothrombin time with the log vitamin dose, give closely parallel lines. The tests with honey diluted with equal volume of water administered directly to the prothrombin deficient chicks resulted in little evidence of vitamin K activity, confirming the results of the previous test cited in connection with series A. The clotting times observed are outside of the range of correct assay of vitamin K by this procedure.

Summary. Buckwheat, alfalfa and mixed honeys when fed incorporated in basal ration to vitamin K depleted chicks were found to possess a definite antihemorrhagic activity equal to approximately 0.25 μg of 2-methyl-1,4 naphthoquinone per gram of honey. The antihemorrhagic activity of honey was greatly lowered when honey was administered in an aqueous solution directly to the vitamin K depleted chicks.

It was demonstrated that the antihemorrhagic activity of 2-methyl-1,4 naphthoquinone is reduced about 3 times when fed in aqueous suspension. The antihemorrhagic activity of alfalfa hay in water suspension was practically nil while with an addition of 2 drops ethyl laurate, the prothrombin time was quite satisfactory.

This work was made possible through the research grant from W. F. Straub and Co., Chicago, Illinois. Grateful acknowledgment is made to Dr. H. J. Sloan for supplying chicks for the experiment and for permitting the use of brooders and other facilities.

[3] Greaves, J. D., and Schmidt, C. L. A., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 43.

[4] Dam, H., and Glavind, J., *Acta Medica Scand.*, 1938, **96**, 108.

14164

Effect of Sympathicolytic and Other Agents on Toxicity of Digitalis in Cats.

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Wegria *et al.*[1] note that ventricular fibrillation caused by toxic doses of digitalis in the dog is of a different type than that evoked by electric stimuli or coronary occlusion and that ventricular fibrillation accordingly may be induced by more than one mechanism. The present study offers proof that the type elicited by digitalis differs also from that caused by epinephrine, chloroform or other adrenergic agents.

A number of substances have been found effective against fibrillation provoked by treatments other than digitalization.[2-9] Of these, the 2 sympathicolytics, piperidinomethylbenzodioxane (933F) and diethylaminomethylbenzodioxane (883F), and procaine are most certain. Their mode of action is not satisfactorily explained; but slitting the pericardium produces the same result,[10] which suggests that a sudden rise in intracardiac pressure may be the precipitating factor for fibrillation. While the cardiac dilatation which occurs terminally in digitalis intoxication is considered a result rather than a cause of arrhythmia and fibrillation, a test of these agents was thought warranted. Recent introduction of potent digitalis derivatives[11] and revival of interest in rapid digitalization[12] have increased therapeutic hazards, for the absence of more frequent clinical accidents is a measure of skill in administering digitalis rather than of the margin of safety of the agent.

Tr. digitalis was administered as in a cat assay method.[13] One cc/kg of a 1:15 dilution was rapidly injected intravenously every 5 min in 33 cats lightly anesthetized with ether, until the heart stopped. Phases of the digitalis effect were followed by frequent auscultation. The test agents were dissolved in the diluted tincture and their administration was usually begun after the heart showed definite digitalis effects. In some instances, as noted in Table I, these agents were given from the first digitalis injection, and in 2 cats saline solutions of 933F or quinidine sulfate were given at 5 min intervals prior to digitalization.

Haag[14] has shown that kittens are similar to cats in their resistance to lethal effects of digitalis, and 6 kittens were distributed proportionately in the 5 groups. Cats were also divided approximately equally as to sex, in each group. Neither weight nor sex was related to toxicity of digitalis, although the endpoint was less sharp in kittens because fibrillation apparently took a longer course than in cats. Hearts were removed after death, opened, trimmed, washed, blotted dry and weighed. In g/kg, the heart weight/body weight ratios and their standard errors were:

1 Wegria, R., Geyer, J. H., and Brown, B. S., *J. Pharmacol.*, 1941, **71**, 336.

2 Hermann, H., and Jourdan, F., *C. R. Soc. Biol.*, 1931, **106**, 1153.

3 Shen, T. C. R., and Simon, M. A., *Arch. internat. Pharmacodyn.*, 1938, **59**, 68.

4 Shen, T. C. R., *Arch. internat. Pharmacodyn.*, 1938, **59**, 243; 1939, **61**, 43.

5 van Dongen, K., *Arch. internat. Pharmacodyn.*, 1938, **60**, 206; 1939, **62**, 261; 1939, **63**, 88; 1941, **66**, 41.

6 Shen, T. C. R., and Marri, R., *Arch. internat. Pharmacodyn.*, 1940, **64**, 58.

7 Allende, I. M., and Orias, O., *Rev. Soc. Argentina Biol.*, 1940, **16**, 467.

8 Wiggers, C. J., and Wegria, R., *Am. J. Physiol.*, 1940, **131**, 296.

9 Beck, C. S., *Am. J. Surg.*, 1941, **54**, 273.

10 Dautrebande, L., and Charlier, R., *Arch. internat. Pharmacodyn.*, 1941, **66**, 257.

11 Council on Pharmacy and Chemistry, *J. A. M. A.*, 1942, **119**, 1025.

12 Gold, H., Kwit, N. T., Cattell, McK., and Travell, J., *J. A. M. A.*, 1942, **119**, 928.

13 Gold, H., Cattell, McK., Kwit, N. T., and Kramer, M., *J. Pharmacol.*, 1941, **73**, 212.

14 Haag, H. B., and Corbell, R. L., Jr., *J. Pharmacol.*, 1940, **68**, 45.

TABLE I.
Effect of Various Agents on Toxicity of *Tr. digitalis* in Cats.

Agent	Single doses, mg/kg	Time of injection single doses (digitalis inj. No.)	Total dose, mg/kg	Lethal dose of *Tr. digitalis* in cc/kg	
				Individual cats	Mean ± Std. Error
None	—	—	—	.60, .60, .67, .73 .73, .80, .80, .80 .87, .87, .93, .93	.78 ± .03
Procaine	1	9-14	6	.93, .93	
	2	9-11	6	.73	
	3	9-11	9	.73	.77 ± .07
	3	9-12	12	.80	
	10	6-7	20	.47	
933F	1	4-11	8	.73	
	1	4-12	9	.80	
	1	1-11	11	.73	.78 ± .01
	2	8-12	10	.80	
	2	1-12	24	.80	
	—	2x5 mg + 5x2 mg prior inj.	20	.80	
883F	1	4-11	8	.73	
	1	1-12	12	.80	.79 ± .06
	1	1-14	14	.93	
	2	8-10	6	.67	
Quinidine	2.5	1	2.5	.07	
	2.5	6-8	7.5	.53	
	2.5	6-10	12.5	.67	.40 ± .14
	2.5	1-10	25	.67	
	—	4x2.5 mg prior inj.	10	.07	

4.61 ± 0.12, 4.67 ± 0.41, 4.62 ± 0.17, 4.36 ± 0.24 and 4.61 ± 0.32 for groups treated with digitalis alone, and digitalis + procaine, 933F, 883F, and quinidine, respectively.

Results are noted in Table I. None of the drugs except quinidine produces a difference in the lethal dose of digitalis. Results with quinidine are most variable: 1 cat, not included in Table I, died after administration of 2.5 mg/kg alone; another from 2.5 mg/kg in 0.067 cc/kg of Tr. digitalis; and another following 10 mg/kg before 0.067 cc/kg of tincture. The lethal dose of quinidine alone is stated[15] to be 80-100 mg/kg when given intravenously in cats but idiosyncrasy is common. With 933F and 883F, the higher doses given approach the lethal range; the former particularly causes light narcosis, so that ether anesthesia is not required during the period of digitalization when it is given. A cat treated with a single injection of 10 mg/kg of 933F alone died of respiratory failure when ether anesthesia was continued, while another received 20 mg/kg and survived when ether anesthesia was promptly discontinued; consequently the depth of anesthesia was closely controlled in digitalized cats receiving 933F or 883F.

Cardiac failure was the cause of death in all cats receiving digitalis, with the exception of 1 cat treated with 20 mg/kg of procaine during digitalization which died of respiratory failure. The lethal dose of procaine is 55-60 mg/kg in cats on intravenous administration.[15] In the remaining 32 cats, the symptoms were entirely those of digitalis: bradycardia followed by increasing tachycardia, arrhythmia and fibrillation. Despite the failure of procaine, together with the other agents, to increase the lethal dose of digitalis, small doses elicited a definitely beneficial effect on the arrhythmia preceding fibrillation in the later extravagal phases of the digitalis action. This beneficial effect of procaine was of short duration, never exceeding 2 min after each

[15] Sollmann, T., and Hanzlik, P. J., *Fundamentals of Experimental Pharmacology*, J. W. Stacey, San Francisco, 1939.

injection, and was evoked as well by single doses of 1 mg/kg as by 3 mg/kg. While procaine had an inappreciable effect on toxicity of digitalis under the conditions of the experiment, it might be of at least transient beneficial effect under other conditions.

Summary. 933F, 883F, procaine and quinidine do not increase tolerance of cats to lethal effects of digitalis. Ventricular fibrillation induced by digitalis overdosage is of a different type than that elicited by epinephrine or related agents.

14165

Fatal Loss of Plasma Volume After Lymph Heart Destruction in Toads.

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In certain cases of shock and after severe burns there is a tendency toward blood concentration. Determinations of total red cell volume or count per cubic millimeter when compared with determinations of plasma proteins and other plasma constituents suggest that the hemoconcentration is due to loss of whole plasma from the blood vessels into the tissue spaces, where it remains manifest as an edema. Normally, the lymphatic system returns any excess intercellular fluid to the blood circulation.

In batrachians, lymph flow is aided by 4 lymph hearts. In both toads (*Bufo arenarum* Hensel) and frogs (*Leptodactyllus ocellatus*) the 2 anterior and 2 posterior lymph hearts can be easily destroyed by thermocautery. Foglia and his collaborators[1,2,3,4] found that if the operation was complete, death ensued in 4 days. Marked changes were found to occur in the water and electrolyte concentrations of blood, lymph and tissues, beginning at operation and continuing until death. There was an enormous increase in body weight due to water retention (60%). These animals were kept in sinks moistened by dripping water and the water absorbed through the skin was retained in the tissue spaces. Since the interstitial fluid failed to reach the blood stream it could not be eliminated by kidney excretion. A complete bibliography of the problem will be found in the paper by Braun-Menendez and Foglia.[3]

During the course of the experiments by Foglia and Gerschman,[2] some animals without lymph hearts were kept in a chamber with high humidity but no liquid water. These toads died with no gain or loss in body weight.

We thought it worth while to investigate the fluid distribution and concentration in this type of experiment since with diminished urine excretion, most of the changes would involve an internal redistribution of water.

Material and Methods. The toads (*Bufo arenarum* Hensel) were operated in one stage under ether anesthesia. All 4 lymph hearts were destroyed by electrocautery using the technic described in previous papers.[1,3] In operated controls an equal connective tissue and muscle area was burned in a nearby region.

Red cell volume was determined in a 10 cm capillary tube with freshly drawn blood, by centrifugation at 3500 r.p.m. for 20 minutes. Coagulation was prevented by a few grains of dry heparin (Connaught Labs.).

Specific gravity of plasma and lymph were determined by the falling drop technic of Barbour and Hamilton.[5] This was converted

1 Foglia, V. G., *Rev. Soc. Argent. Biol.*, 1939, **15**, 97; *C. R. Soc. Biol.*, 1940, **133**, 153.

2 Foglia, V. G., and Gerschman, R., *Rev. Soc. Argent. Biol.*, 1939, **15**, 113; *C. R. Soc. Biol.*, 1940, **133**, 155.

3 Braun-Menendez, E., and Foglia, V. G., *Arch. Inter. de Pharm. et Therap.*, 1940, **64**, 273.

4 Foglia, V. G., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 598.

5 Barbour, H. G., and Hamilton, W. F., *J. Biol. Chem.*, 1926, **69**, 625.

to grams of protein by subtracting 1.0054 for water and salts from the density and multiplying by 340.1.

Potassium was determined in plasma by the micro method of Truszkowski and Zwemer[6] and in tissues by the method of Marenzi and Gerschman.[7]

Tissue water was studied by drying a chopped mass from many animals, or by small pieces rapidly weighed on a micro torsion balance and then dried to constant weight, and also in a few cases by recovery of water distilled off from a mass of chopped tissue.

Tissue sections were made by frozen and paraffin methods and stained with hematoxylin and Sudan III or eosin.

Constant humidity was maintained in a large wide-mouthed, glass bottle fitted with a 2-hole rubber stopper. Air was evacuated through one tube connected to an ordinary water suction pump. Air entering the chamber through the other tube was first bubbled through water, thus ensuring constant saturation regardless of temperature. By placing the container on its side, a larger number of toads could be accommodated.

Discussion of Results. The first requirement was confirmation of the constancy of body weight, since this was the factor that determined the fact of an *internal* redistribution of water. The weights of individual toads in the chamber was so constant that individual listing would be an unnecessary waste of tabular space so group weights are given in Table I. In the presence of liquid water, absorption through the skin produced large gains and the animals are listed separately.

In the blood and lymph studies (Fig. 1) the animals were tested for packed red blood cell volume, plasma and lymph protein and plasma and lymph potassium. The figures given are averages of separate determinations in 3 to 5 animals. The most important change appears to be the steady increase in red cell volume. For some hours before death it was extremely difficult to obtain samples from the heart or aorta. One can deduce the plasma-free state and red cell congestion in the peripheral vessels.

The water content of the liver and muscle tissue of operated animals in constant humidity

TABLE I.
Body Weight of Toads After Lymph Heart Destruction.

Controls In humid chamber Group weights (g)			After lymph heart destruction					
			In humid chamber* Group weights (g)			In the presence of running water Individual weights (g)		
16 ♂	4/28	5/5	4 ♂	5/6	5/7	3/31	4/1	4/2
	2335	2320		550	550	215	255	265
						165	200	240
16 ♂	5/7	5/10	8 ♂	5/7	(8 hrs later)	155	180	—
	1840	1880		1130	1120	125	150	160
5 ♂	5/10	5/13	10 ♂	5/10	5/13	7/3	7/4	7/5
	560	570		1180	1180	102	127	140
						105	120	130
16 ♂	5/15	5/19	8 ♂	5/19	5/21	104	122	135
	1810	1800		970	960	105	122	130
						110	130	145
4 ♂	6/26	6/28				103	115	120
	372	370				85	110	122
						106	125	130
						100	123	125
						100	122	125
						72	95	95
						All toads gained wt within 24 hr of operation.		

* Individual animal weights of controls and operated animals in humid chamber showed remarkable constancy.

[6] Truszkowski, R., and Zwemer, R. L., *Biochem. J.*, 1937, **31**, 229.

[7] Marenzi, A. D., and Gerschman, R., *Rev. Soc. Argent. Biol.*, 1932, **8**, 38.

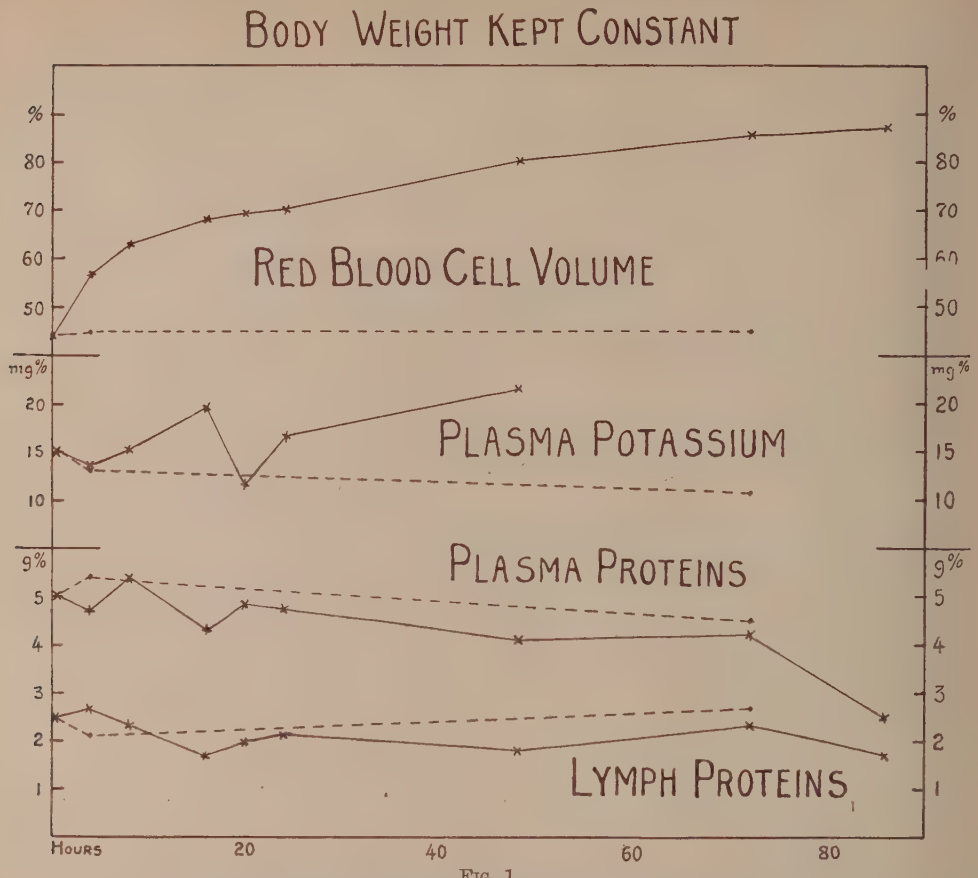

FIG. 1.

Blood and Lymph Changes After Lymph Heart Destruction.

In the graph each point is an average of separate determinations in 3 to 5 animals. Solid lines represent data from experimental animals. Broken lines, data from "dummy-operated" controls run under identical conditions.

did not change significantly (Table II), whereas in the presence of water there was an increase in the per cent of tissue water.

The plasma protein concentration remained constant, thus indicating that whole plasma was lost through the capillary walls. The fact that the lymph protein did not rise can be explained on the basis of proportion of plasma extravasated to the total amount of tissue fluid already present in the animal. A 100 g toad with 6% blood (of which 55% is plasma) would have 3.5 cc of plasma. When the plasma volume falls to 15% of the blood (Fig. 1) there has been a calculated loss of 2.6 cc. This would give a negligible change in the total lymph protein concentration.

However, in the presence of running water there was a marked fall in the protein content of both plasma and lymph. This is in accord with the great water retention noted by Foglia[4] and confirmed by the present experiments.

The plasma and lymph potassium showed a tendency to increase in constant humidity. This increase reached approximately 100% when the animals were in contact with water, the potassium being derived from erythrocytes and other cells as found by Foglia.

Sections of tissues by both frozen and paraffin technics showed no significant differences in cell size between the controls and the operated animals either humid or in contact with liquid water.

TABLE II.
Water Content of Tissues.

Group	Time, hr	Liver	Muscle
Control			
A	0	68.8	77.1
B	0	67.7	75.2
C	0	—	77.6
Without lymph hearts, humidity constant			
B	48	68.5	75.9
C	48	70.3	75.3
C	86	71.7	74.9
Without lymph hearts + water present			
A	24	70.2	81.5
C	30	72.6	83.4
B	48	72.4	80.6
B*	48	68.7	78.0
C	48	73.7	80.8

A. Data from Foglia and Gerschman.

B. Data from Dr. Mazzocco on mixed samples from many animals.

B*. Special water recovery method.

C. Data averaged from many single determinations in present experiments.

Conclusions. Toads without lymph hearts kept in constant humidity showed no change in body weight, whereas other animals kept in the presence of water showed a gain in weight of as much as 20% daily. With a constant body weight the fluid redistribution can be assumed to be internal. In both series of experiments there was a marked increase in red cell volume to a point where flow even in large vessels was impeded.

The fall in plasma and lymph protein, and the rise in body fluid, potassium content and tissue water are much greater when water is absorbed through the skin by contact.

When the normal flow of fluid from plasma to tissue space to lymph channels to vascular system is blocked by lymph heart destruction, there is a marked increase in the interstitial fluid at the expense of the plasma. The failure of this edema fluid to return to the blood vessels in these experiments is a primary and uncomplicated cause of death.

The senior author wishes to thank Prof. B. A. Houssay for laboratory facilities extended to him while carrying on these experiments in Buenos Aires, and the John S. Guggenheim Memorial Foundation for making the visit possible.

14166

The Effect of Phosphatides on Utilization of Vitamin A and Carotene.

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The problem of the utilization of carotene and vitamin A as influenced by the oil or fat in the diet has been investigated repeatedly during recent years.

Sherman[1] studied the effect of various oils on the utilization of carotene and found that the addition of 0.1 ml of cottonseed oil daily and especially soybean oil to the diet greatly improved the growth response of vitamin A deficient rats receiving 1 and 2 μg carotene daily.

Quackenbusch, Cox and Steenbock[2] reported that tocopherol promoted the response to carotene and concluded that tocopherol functioned in the intestinal tract as an antioxidant.

Sherman[3] reported that carotene was destroyed in the intestinal tract in the absence of tocopherol.

Quackenbusch, Cox and Steenbock[4] provided further evidence that tocopherol is essential for the utilization of carotene in the intestinal tract but reported even better results with a soybean oil distillate.

1 Sherman, W. C., *J. Nutrition*, 1941, **22**, 153.

2 Quackenbusch, F. W., Cox, R. P., and Steenbock, H., *J. Biol. Chem.*, 1941, **140**, civ.

3 Sherman, W. C., *Fed. Proc. Soc. Biol. Chem.*, 1942, **1**, 134.

4 Quackenbusch, F. W., Cox, R. P., and Steenbock, H., *J. Biol. Chem.*, 1942, **145**, 169.

As it is known that the antioxidant activity of tocopherol *in vitro* is greatly influenced by cephalin[5] it seemed advisable to investigate this problem further. The evidence presented by our experiments indicates that this synergism between cephalin and tocopherol exists also *in vivo* but that other factors may be involved in the utilization of carotene and vitamin A by the rat.

Experimental. Female albino rats, Sherman strain, from our laboratory colony, 28 to 30 days old, weighing approximately 60 to 66 g, were kept in individual metal cages provided with raised screen floors. The basal diet used had the following percentage composition: casein Labco, 20; cerelose, 72; salts, Osborne and Mendel, 4; cottonseed oil, 4. This diet was supplemented with thiamine 2 mg, riboflavin 4 mg, pyridoxine 4 mg, nicotinic acid 1 mg, choline 500 mg and 200 mg of Viosterol per kilogram of diet. Synthetic calcium pantothenate was given orally, 100 μg daily. The rats were weighed daily during the depletion period and 3 times a week during the 4-week period when the test substances (phosphatides and soybean oil) were included in the diet. The purified diets and mixtures were freshly prepared each week and stored at 4°C. Food consumption was determined.

Discussion. Although our basal diet was similar to the diet used by Sherman, Steenbock, *et al.*, we failed to get the expected growth response with the rats receiving 2 U.S.P. units carotene or vitamin A and a diet containing 4% cottonseed oil which should have been sufficient to provide the necessary tocopherol. However, our diet differed from those mentioned before in that both authors used yeast or rice bran extract as a source of the B complex in their diet while we used the synthetic vitamins mentioned above. The striking response of our rats to 1% soybean phosphatides,* and to a certain degree to 0.3% soybean oil, indicates the presence of an unknown factor, possibly also present in yeast or the B complex and essential for the utilization of vitamin A and that this factor is not present in cottonseed oil.

That factors of the B complex may be essential for the utilization of vitamin A was proved by Popper[6] who showed that choline deficiency interferes with vitamin A utilization. However, in our experiments, enough choline was provided to exclude this possibility. It may be noted that the presence of a hypothetical factor for the utilization of vitamin A and carotene has been suspected before[7] and it was claimed that palm kernel meal, coconut cake and acetone-extracted herring roe are rich in this factor.

It can be seen from Chart 1 that the vitamin A-depleted rats gave essentially the same type of growth response with 2 units of carotene as with 2 units of vitamin A (cod liver oil) when 1% of soybean phosphatides was added to the diet. As these soybean phosphatides contain considerable amounts of soybean oil (up to 30%) two groups of rats were given the equivalent amount of soybean oil (0.3%) instead of the phosphatides in the diet. Although the rats receiving soybean oil plus 2 units of vitamin A showed a gain in weight, they failed to equal that obtained with the phosphatides. After the third week the rats receiving soybean oil instead of soybean phosphatides started to lose weight while those receiving phosphatides continued to gain. Even more striking was the fact that the animals receiving phosphatides were in much better condition at the end of the 4-week test period than the rats receiving only soybean oil. The fur of the soybean oil rats appeared shaggy and dry while that of the phosphatides rats was sleek and glossy.

The above experiments were repeated substituting 4% soybean phosphatides in the place of 1% phosphatides in the diet. The increased intake of soybean phosphatides did not appreciably change the gain in weight.

In addition, two groups of vitamin A de-

[5] Swift, C. E., Rose, W. G., and Jamieson, G. S., *Oil and Soap*, 1942, **19**, 176.

* We are indebted to the American Lecithin Co., Elmhurst, L.I., N.Y., for furnishing us with commercial soybean phosphatides. These soybean phosphatides are composed of about 20 to 25% lecithin, 25 to 30% cephalin fraction, including some carbohydrate, about 15% inositol phosphatides and a carrier of 30% soybean oil.

[6] Popper, H., and Chinn, H., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 202.

[7] Morton, R. A., *Ann. Rev. Biochem.*, 1942, **11**, 371.

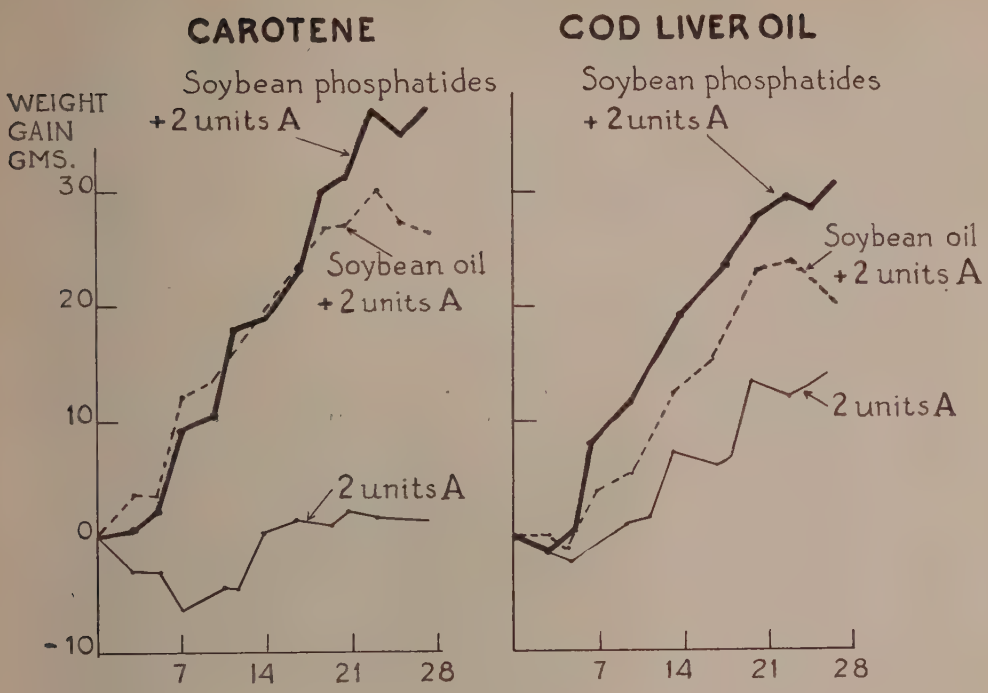

CHART 1.

pleted rats were given soybean phosphatides without vitamin A. These rats lost weight even more rapidly than those receiving the basal diet without vitamin A. This response may be due to the strong lipotropic effect of soybean lecithin.

Summary. Vitamin A-depleted rats fed a vitamin-free diet, supplemented with synthetic B vitamins and 2 U.S.P. units of vitamin A in the form of carotene, failed to gain weight in spite of the presence of 4% cottonseed oil in the diet. Satisfactory growth was obtained when soybean phosphatides (1%) were added to the diet. An amount of soybean oil (0.3%) equivalent to that contained in the soybean phosphatides used also promoted growth but to a lesser degree. Rats fed the vitamin A-deficient diet supplemented with soybean oil and 2 units of vitamin A appeared in poor condition and began to lose weight after the third week.

Substitution of cod liver oil in place of carotene as the source of vitamin A in the diet, with addition of soybean oil or soybean phosphatides resulted in a growth response similar to that obtained with carotene. However, cod liver oil alone also caused some gain in weight.

In the presence of soybean phosphatides, carotene was as well utilized as vitamin A under the conditions of our experiment.

14167

Acute Toxicity for Rats and Mice of 2-Ethyl Hexanol and 2-Ethyl Hexyl Phthalate.*

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Since 1869 the phenomenon of increasing toxicity of alcohols with increasing molecular weight has been referred to as Richardson's Law.[1] Considerable data in detail on many alcohols have appeared in the last twenty years, and, in general, the results confirm Richardson's observations.[2-12] Secondary alcohols have been shown to be less toxic than the corresponding primary alcohols. However, no investigation has been sufficiently comprehensive to include simultaneous measurements on straight and branched chain, primary and higher alcohols.

No published data are available on the toxicity of 2-ethyl hexanol. However, it appears that this branched chain octanol has, in rats, about the same order of toxicity as ethyl alcohol in cats (intraperitoneal administration). Ethyl hexanol given into the stomach seems to be only one-sixth as toxic in rats as ethyl alcohol, given the same way, is in rabbits.

Method. The acute toxicity of ethyl hexanol was measured on groups of male and female adult albino rats. Two routes of administration were used (a) intraperitoneal injection, and (b) stomach tube administration. The 2-ethyl hexanol was taken from a 3 kg sample of Eastman Kodak Company, practical grade.

Intraperitoneal Injection in Rats. In a preliminary experiment groups of 5 rats each were injected with various doses of ethyl hexanol intraperitoneally. The lethal dose for the average rat lay between 0.1 and 0.2 cc. On the basis of this information, groups of about 15 rats each were given doses ranging from 0.10 to 0.24 cc of ethyl hexanol (Table I, A). The percentage mortality-dosage curve was a typical s-shaped curve without undue scatter. The LD 50, calculated by the method of Bliss, was 0.078 cc per 100 g rat or 0.65 g per kg body weight. This represents a moderate toxicity.

Rats given ethyl hexanol intraperitoneally promptly developed an irregular gait and exhibited dragging of the hind legs. In the next few minutes after high doses, the breathing became gasping in character, although cyanosis was not noted. Insensibility rapidly followed and even on the lower doses (0.02 cc per rat) all the rats were sound asleep in 10 minutes. It appeared that ethyl hexanol has marked anesthetic properties.

Stomach Tube Administration to Rats. In a preliminary experiment on groups of 5 rats each, it was found that the lethal dose lay between 0.6 and 1.0 cc. Consequently large groups of rats (Table I, B) were given doses as shown. The percentage mortality-dosage curve is a smooth s-shaped curve and the LD 50 calculated according to the method of

* This work was supported in part by a grant from the Carnegie Corporation of New York.

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TABLE I.
Acute Toxicity of 2-Ethyl Hexanol in Rats and Mice.

No. rats	Avg body wt, g	Dose, cc	No. dead	% mortality
	A. Intraperitoneal Injection (101 rats).			
5	177	.02	0	0
5	193	.05	0	0
15	269	.10	2	13
15	234	.14	3	20
15	197	.16	6	40
15	208	.20	13	87
16	206	.24	14	88
10	345	.40	7	70
5	217	.40	12	100
LD 50 (probit kill — 5.0) = 0.078 cc per 100 g rat.				
	B. Stomach Tube Administration (85 rats).			
5	183	.05	0	0
5	207	.20	0	0
15	230	.60	3	20
15	189	.80	9	60
15	184	1.0	11	73
15	179	1.6	14	93
15	294	2.0	15	100
LD 50 (probit kill — 5.0) = 0.39 cc per 100 g rat.				
	C. Intraperitoneal Injection (102 mice).			
5	22	.005	0	0
5	24	.010	0	0
15	23	.015	2	13
15	23	.020	9	60
17	23	.025	16	94
15	23	.030	15	100
30	23	.05+	30	100

Bliss is 0.39 cc per 100 g rat or 3.2 g per kg body weight. Thus for the average rat, about 5 times as much ethyl hexanol must be given orally to produce death than is required intraperitoneally.

Intraperitoneal Injection in Mice. In Table I C are given the results from the administration of various doses of 2-ethyl hexanol intraperitoneally to mice. The ethyl hexanol was administered in a 1:10 dilution in mazola. This vehicle caused no apparent change in the symptoms produced by the ethyl hexanol; the mice promptly went to sleep as did the rats which received undiluted ethyl hexanol. The dose to kill the average mouse is of the order of 0.018 cc or 0.78 g per kg body weight. The lethal dose for mice is of the same magnitude as that for rats following intraperitoneal injection.

Acute Toxicity of 2-Ethyl Hexyl Phthalate. The toxicity of phthalates has been previously shown to decrease with increasing molecular weight of the alkyl component.[13] That the toxicity is also a function of the nature of the alkyl moiety is indicated from the current finding that 2-ethyl hexyl phthalate is much less toxic than di-octanol-2 phthalate.

Stomach Tube Administration to Rats. In Table II A are given the data on a total of 50 rats given various doses of 2-ethyl hexyl phthalate. No fatalities were obtained. "Diarrhea" was frequently produced by the largest doses. However, the drug caused a sweeping out of the gastro-intestinal tract and not the production of liquid feces. Twenty-four hours later the gastro-intestinal tracts contained formed feces, and the feces pellets which had accumulated were firm not soft. The maximum dose of 34 g per kg body weight corresponds to a dose of over 2 liters for a 70-kg man, and indicates the extremely low toxicity of ethyl hexyl phthalate in rats.

On autopsy the organs in the peritoneal cavity were normal-appearing. The only peculiarity was the engorgement of the stomachs which seemed perhaps twice as large as filled

[13] Hodge, H. C., Goldstein, M. R., and Wrightington, H., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 471.

TABLE II.
Acute Toxicity of 2-Ethyl Hexyl Phthalate in Rats and Mice.

No. rats	Avg body wt	Dose g/kilo	Effects
	A. Stomach Tube Administration (50 rats).		
15	136	34.5	Diarrhea
15	137	13.7	"
5	200	4.5	"
5	214	2.8	None
5	205	2.0	"
5	189	1.1	"
	B. Intraperitoneal Injection (50 rats).		
15	198	23.8	Diarrhea
15	196	19.2	Some diarrhea
5	155	18.1	?
5	149	12.7	None
5	159	5.7	"
5	159	3.1	"

C. Intraperitoneal Injection (65 mice).

No. mice	Avg body wt	Dose g/kilo	No. dead	% mortality
25	19	50	3	12
20	22	86	3	15
20	22	128	1	5

stomachs ordinarily appear. Perhaps the cause lay in a temporary starvation following the administration of ethyl hexyl phthalate which was then followed by a period of gorging. However, some slowing of the pyloric emptying may have resulted.

Intraperitoneal Injection in Rats. In Table II B data are given on the intraperitoneal injection of ethyl hexyl phthalate to a total of 50 rats. No deaths were observed although one group of 15 rats received about 24 g of ethyl hexyl phthalate per kg body weight. The only effects noted were evidences of diarrhea.

On autopsy the peritoneal cavities contained a milky watery emulsion and the livers seemed to be unusually large and firm. Consequently a group of 5 rats were killed on the third day after receiving ethyl hexyl phthalate, 5 additional rats were killed two days later, and 6 rats were killed 10 days later. The average liver weights were 8.8, 7.8, and 7.3 g, respectively. Since the last figure was nearer the average liver weight in normal rats, it may be assumed that intraperitoneal injection of ethyl hexyl phthalate produced some sort of toxic reaction in the livers, which effect was subsiding during the 10-day period. Liver sections were taken from each rat and stained with hematoxylin and eosin. The following report was submitted by J. R. Carter of the Department of Pathology:

"All sections show definite cytoplasmic changes consisting of increased swelling of cells with marked granularity and moderate dissolution and degeneration. All areas have a decided foamy appearance—both portal and central areas being equally involved. In many areas, the sinusoids are obliterated by the swollen cells. No areas of necroses are seen and nuclear changes, if any, are minimal. The bile canliculi are normal.

"Impression: No specific diagnosis can be attached to these findings except, perhaps that of cloudy swelling. The process is diffuse and the vacuolated areas producing the foamy appearance is due to some substance which will not stain with hematoxylin and eosin."

Intraperitoneal Injection in Mice. Groups of 20 to 25 mice each were given various doses of ethyl hexyl phthalate by intraperitoneal injection (Table II, C). Of the 25 mice receiving 1 cc, 3 died; of the 20 mice receiving 2 cc, 3 died; and of the 20 mice receiving 3 cc, one died. On the largest dosage the mice received 128 g per kg body weight, which would correspond to more than 9 liters for a 70-kg man. The fact that the mortality does not increase with the dosage emphasizes the low toxicity of ethyl hexyl phthalate in mice.

Summary. 1. The acute toxicity of 2-ethyl hexanol following intraperitoneal injection in 101 rats has been measured. The LD 50 was

found to be of the order of 0.08 cc per 100 g rat. From the administration of 2-ethyl hexanol by stomach tube to 85 rats, the LD 50 has been shown to be of the order of 0.39 cc per 100 g rat.

2. A marked anesthetic quality of 2-ethyl hexanol was observed.

3. 2-ethyl hexyl phthalate given by stomach tube to 50 rats in doses of 1 to 34 g per kg body weight produced no deaths. 2-ethyl hexyl phthalate has a very low order of toxicity in rats.

4. 2-ethyl hexyl phthalate given intraperitoneally in doses of 1, 2, and 3 cc respectively, to 65 mice resulted in the death of 7. However, only one mouse in a group of 20 was killed by the largest dose (128 g per kg body weight). 2-ethyl hexyl phthalate has a very low order of toxicity in mice.

14168 P

Probable Mechanism by Which Somatic Changes in Certain Emotional States Are Mediated.*

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Somatic changes commonly occur as the result of emotional reactions such as fear, tension and anxiety. Among the somatic changes are sweating, tachycardia, elevation of blood pressure, leucocytosis, increased intestinal peristalsis and alterations in metabolism of glucose and in skin temperature. Results of investigations on certain of these changes were reported by Diethelm[1] on glucose metabolism, Mittelmann and Wolff[2] on skin temperature, and Milhorat, Small and Diethelm[3] on leucocytosis.

In the present investigations an attempt was made to understand the mechanism or mechanisms by which these somatic changes are mediated. Many of the changes resemble those induced by the administration of adrenalergic and cholinergic drugs. In fact, Cannon and de la Paz[4] observed that blood withdrawn from the suprarenal veins of cats during periods of fear had adrenalergic effects on a surviving strip of rabbit intestine, whereas blood drawn while the animals were quiet had no such effect. The method used in the present studies represents a modification of the procedure of Cannon and is described below. The method of determination of adrenalin in the blood by colorimetric means,[5] was found unsatisfactory for our purposes.

Methods and Materials. Healthy adult rabbits were killed by a blow on the head. The intestines were removed immediately and with careful handling were washed in saline. A loop of intestine was suspended in Ringer-Tyrode's solution, attached to a recording lever and the contractions were recorded on a kymograph. The solution was gently agitated throughout the experiment by means of a stream of air bubbles. The bottle containing the muscle strip and Ringer-Tyrode's solution was suspended in a water bath at 37°C.

The blood was drawn from the patient's cubital vein, mixed immediately with heparin in a small Erlenmyer flask and within a period

* Aided by a grant from the John and Mary R. Markle Foundation.

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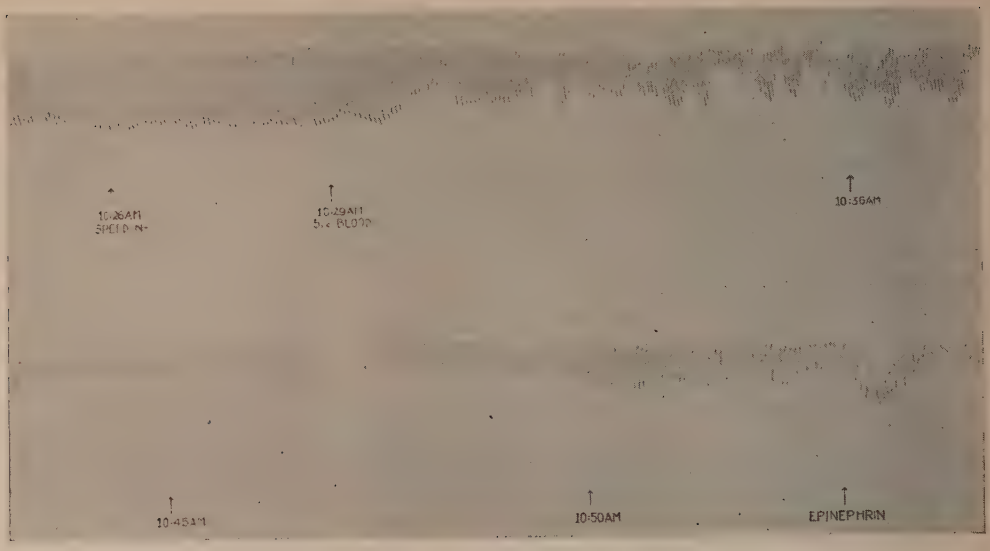

FIG. 1.

of 3 minutes, 5 cc were added to the Ringer-Tyrode's solution. At the conclusion of every experiment the muscle was tested by adding acetylcholine or adrenalin to the solution. The amounts of heparin used were found to be without influence on the spontaneous activity of the muscle or on the response to the pharmacologic agents used.

The subjects were patients with psychiatric disorders who were resident in the Payne Whitney Psychiatric Clinic; members of the hospital staff and patients without psychiatric illness were used in the control experiments.

Observations and Discussion. Blood samples of patients displaying marked anxiety and fear, which often reached the intensity of panic, altered the rhythmic contractions of the rabbit intestine. Usually the amplitude was reduced with incomplete relaxation of the muscle strip so that the base line was elevated. The uniformity and rhythmicity of the contractions was always disturbed and the frequency of contractions was often decreased. Blood that was withdrawn while the subjects showed anxiety induced alternating phases of larger and smaller contractions indicating a superimposed gross rhythm. (Fig. 1.)

These effects were not the relatively simple responses observed when adrenalin or acetylcholine was used. The changes were mixed in nature but generally tended to resemble those of the cholinergic drugs since the base-line

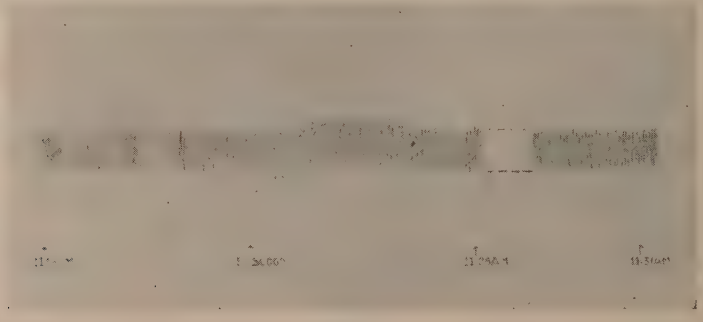

FIG. 2.

usually was elevated. Experiments repeated on patients who previously had shown anxiety or fear and whose blood had had the effects described, showed the blood to be without this pharmacologic activity after the emotional state had subsided (Fig. 2).

The nature of the substance in the blood that induces these changes is not yet known but the substance appears to be labile and disappears almost completely when the blood is allowed to stand for periods of from 15 to 20 minutes. However, the effects produced on the intestinal loop may persist for periods as long as 30 minutes when the blood has been used within 2 to 3 minutes after withdrawal. When the Ringer-Tyrode's solution containing the blood is replaced by fresh solution, normal muscular contractions return within from 5 to 8 minutes. This suggests that the substance can easily be washed out of the intestinal loop.

These observations indicate that the blood of patients showing fear and anxiety contains a substance having definite pharmacologic effects on the surviving intestinal muscle of the rabbit, and offer a possible explanation of the mechanism by which somatic changes in certain emotional states are mediated.

14169

Improved Technic for Demonstrating the Presence of *Streptococcus salivarius* on Eating Utensils.

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In a previous communication,[1] the use of a substrate containing sucrose, potassium tellurite and crystal violet for the selective isolation of "typical" strains of *Streptococcus salivarius* from washed restaurant utensils was reported. This medium demonstrated that common contaminants of drinking glasses, such as the spore-forming bacilli, coliform bacteria and staphylococci, were effectively inhibited, while streptococci apparently grew unhindered. The "typical" strains of *Streptococcus salivarius,* previously described by Niven *et al.*[2] grew by forming distinctive mucoid colonies (Plate I) and could be easily distinguished from other cocci which might occur. The number of isolations obtained during routine use of the medium were less than anticipated, indicating that the combination of potassium tellurite and crystal violet might be somewhat inhibitory to the desired organisms.[3] Such results might be expected in view of the relatively small inocula derived from glasses which have undergone washing.

Additional experiments have been undertaken to determine the selective inhibitory activity of 5 solid substrates. The media of Niven *et al.*[4] and of Stiles and Chapman[5] (minus crystal violet but with added sucrose, 1%) were compared with our previously reported basal substrate containing 3 combinations of inhibitors, *viz.,* potassium tellurite (0.03%), potassium tellurite (0.03%) + crystal violet (1:500,000), and potassium tellurite (0.02%) + sodium azide (0.02%). Twenty-four-hour broth cultures of 14 bacterial species, representative of those likely to occur on eating utensils, were streaked upon each of these media and incubated for 72 hours at 37°C. Adequate controls on non-inhibitory media were inoculated simultaneously. Results were recorded on a comparative basis using growth on the control plates as the standard of comparison. The data in

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2 Niven, C. F., Smiley, K. L., and Sherman, J. M., *J. Bact.*, 1941, **41**, 479.

3 Unpublished data.

4 Niven, C. F., Smiley, K. L., and Sherman, J. M., *J. Bact.*, 1942, **43**, 113.

5 Stiles, M. H., and Chapman, G. H., *J. Bact.*, 1942, **43**, 64.

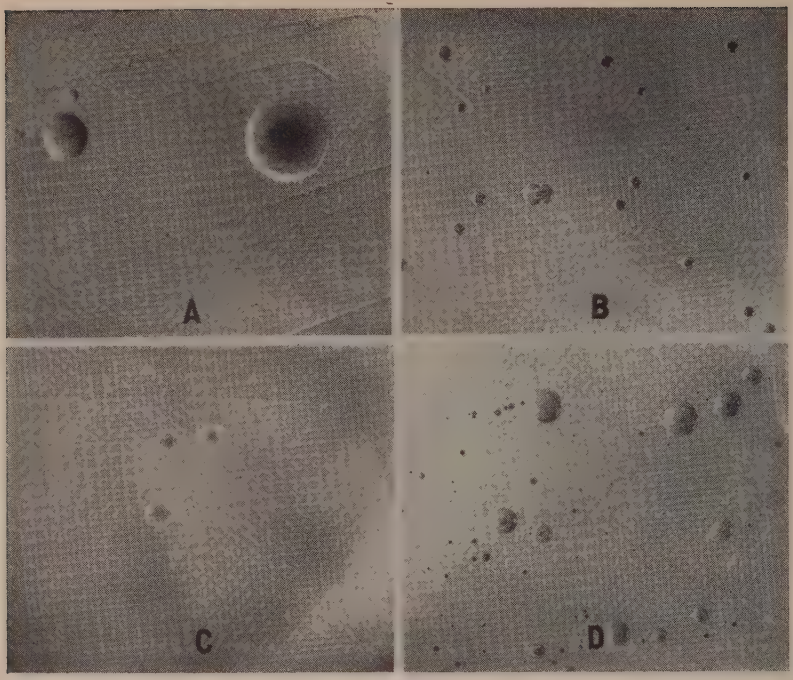

PLATE 1.

A. "Typical" *S. salivarius;* large mucoid colonies. B. Type "B" strain; hard gritty colonies. C. "Atypical" *S. salivarius;* flat. All are pure cultures, 72 hours. D. Direct smear from saliva showing typical and atypical forms. Age, 72 hours. Magnification, 2½ diameters.

Table I show that none of the media employed, with the exception of Medium IV, adequately inhibited the undesirable organisms while simultaneously permitting "typical" *S. salivarius* to grow unhindered.

Experiments to determine the effect of Media I, II and III on small inocula (10 to 200 "cell groups") of *S. salivarius* demonstrated that Media I and II were completely inhibitory. Medium III was non-inhibitory to inocula of *S. salivarius* as small as 25 cells, but its lack of inhibition of other organisms (Table I) eliminated its use for routine analyses. In fact, Walter[6] employed III in a restaurant survey, reporting no recovery of oral streptococci because of surface overgrowth by yeasts and coliform bacteria. Medium IV was also found unsatisfactory since a heavy inoculum of streptococci was required to initiate growth upon its surface. It became apparent that the use of a solid medium in the isolation of streptococci from washed glasses was attended by difficulties, not the least of which was the inability of small inocula of *S. salivarius* to grow well on a medium sufficiently inhibitory to eliminate undesirable organisms. An enrichment medium of some sort was deemed necessary.

A variety of enrichment media were tested and one containing sodium azide was found most satisfactory. Its composition follows:*

Proteose peptone (Difco)	5 g
Yeast extract (Bacto)	5 "
Beef extract (Bacto)	3 "
Glucose (C.P.)	10 "
Sodium azide (1% aq.)	20 ml
Distilled water	1000 "
Adjust to pH 7.0-7.2	

6 Walter, W. G., *J. Bact.*, 1942, **43**, 114.

* Sodium azide is relatively stable to heat and can be added to the medium prior to sterilization. Care must be exercised to maintain the pH near 7.0 to 7.2. It was found that any marked divergence rendered the medium inhibitory to strains of *S. salivarius* employed in these studies.

TABLE I.
Growth of Microörganisms on Media Designed for Selective Isolation of "Typical" *Streptococcus salivarius.*

Bacterial species	Growth on selective media*				
	I†	II	III	IV	V
Escherichia coli	—	—	++	—	±
Aerobacter aerogenes	±	±	±	—	±
Salmonella typhimurium	—	—	+±	—	+
Slow lactose fermenter No. 1	—	—	+	—	+±
Proteus morganii	+	+	+++	—	++
Proteus vulgaris	+++	+++	+++	—	++
Proteus species	++++	++++	++	+	++
Staphylococcus aureus	—	+++	+++	++±	++
Bacillus subtilis	±	+++	+++	—	++
Saccharomyces cerevisiæ	+	++	+++	+±	++
Torula lactosa	+	+	+	+	—
Streptococcus liquefaciens	++	++++	++++	+++	++++
Streptococcus salivarius No. 6	++	++	+++	++	++++
Streptococcus salivarius No. S25D	++	++++	++++	++++	++++
Streptococcus salivarius No. 83	++	++++	++++	++++	++++

* Results recorded on comparative basis with growth on the control = ++++.
† I = RG basal medium plus K_2TeO_3 (0.03%) and crystal violet (1:500,000).
II = RG basal medium plus K_2TeO_3 (0.03%).
III = NSS medium (0.02% NaN_3).
IV = RG basal medium plus K_2TeO_3 (0.02%) and NaN_3 (0.02%).
V = Modified Chapman's medium (0.02% NaN_3).

Experiments to determine the size of inoculum needed to initiate growth in this broth indicated that dilutions as high as 1:1 billion, or an inoculum of approximately 1 to 10 cells of "typical" *S. salivarius,* could initiate growth

TABLE II.
Effect of Inoculum Size on Initiation of Growth in Enrichment Medium Containing Sodium Azide.

Bacterial species	Greatest dilution of inoculum from which growth was initiated. (Avg of 3 trials)	
	Basal medium	Basal medium + NaN_3
S. salivarius S25D*	1 x 10–9	1 x 10–9
S. salivarius S20B*	1 x 10–9	1 x 10–9
S. salivarius No. 6†	1 x 10–9	1 x 10–8
S. salivarius No. 208‡	1 x 10–9	1 x 10–8
Staphylococcus aureus	1 x 10–9	1 x 10–8
Escherichia coli	1 x 10–9	1 x 10–4
Proteus species	1 x 10–9	1 x 10–5

* "Typical" strain.
† "Atypical" strain.
‡ Strain "B."

(Table II). *Staphylococcus aureus, Streptococcus liquefaciens* and an "atypical" (non-gum-forming) *S. salivarius* were also able to grow from minute inocula, whereas heavier inocula of *E. coli* and *Proteus* species were required. Organisms of the type *Proteus* species were particularly troublesome in routine isolations. They were encountered frequently in samples obtained from restaurant glasses and formed voluminous quantities of a mucoid material on sucrose media, readily distinguishable, however, from *S. salivarius.* These results suggest that preliminary cultivation in azide broth would tend to eliminate this group of organisms. In fact, *Proteus* species occurred frequently when direct streaks on solid media were employed, but they were not encountered in 398 field samples using an enrichment technic. Edwards[7] has demonstrated that inocula of *S. mastitis* consisting of 31 to 135 cells eliminated large inocula of *E. coli* when the 2 were grown in association in an azide-containing enrichment broth. Two experiments were conducted in which growth curves were obtained for *S. salivarius* and *E. coli* cultivated simultaneously in the enrichment broth. In both instances a heavy inoculum of *E. coli* was progressively reduced in numbers over a period of 16 hours by a smaller inoculum of *S. salivarius. S. salivarius* exhibited a normal growth curve and was unaltered by the association with *E. coli.*

For field experiments, a simple routine

[7] Edwards, S. J., *J. Comp. Path. and Therap.,* 1938, **51**, 250.

TABLE III.

Incidence of Oral Streptococci on Washed and Unwashed Glasses and in Wash and Rinse Waters as Determined by a Technic Involving Primary Enrichment in an Azide Broth.

Source of sample	No. of samples	Incidence of *S. salivarius*					
		"Typical" strain		Strain "B"		"Atypical" strain	
		No.	%	No.	%	No.	%
Unwashed glasses	44	23	52.3	13	29.5	25	57.0
Washed glasses	238	13	5.5	0	0	93	39.0
Wash water	20	5	25.0	0	0	10	50.0
Rinse water	9	0	0	0	0	4	44.4

technic was developed.† Cotton swabs on 5″ applicators were suspended, through cotton plugs, in 5 ml of phosphate buffer at pH 7.0. For sampling purposes, the swab was slightly moistened in the buffer solution, the rim of a glass streaked vigorously and the swab returned to the buffer solution. In the laboratory, after vigorous agitation, the swab plus 1 ml of the phosphate solution was transferred to 9 ml of the enrichment medium and incubated at 37°C until growth appeared, but not longer than 48 hours. Large loopfuls (4.0 mm) of the cultures were then streaked on tellurite-crystal violet-sucrose agar plates and incubated 2 to 4 days at 37°C. The remainder of the phosphate buffer was used for the inoculation of other media. At the end of the incubation period, the plates were examined for colonies of "typical" *S. salivarius*.

Table III shows results obtained from 44 samples of unwashed glasses. "Typical" *S. salivarius* was recovered from 52% of the glasses tested, the results agreeing with the 45% recovery reported previously.[1] The "B" and "atypical" strains are 2 of *S. salivarius* which do not form mucoid colonies. The exact identity of strain "B" is not as yet established although it possesses several of the characteristics of the Lancefield Group H. The "atypical" strain resembles the "atypical" *S. salivarius* of Sherman.‡

Two hundred thirty-eight washed glasses in 24 different eating establishments were examined by the above procedure. These results are also presented in Table III and show that 5.5% of the glasses tested carried "typical" *S. salivarius*. The presence of "typical" *S. salivarius* on washed glasses demonstrates that the washing procedure employed was ineffective and that a potential chain of infection from the mouth of one individual to that of the next user exists. Although this paper is primarily a report on the development of a procedure, it is worthwhile to note that glassware in 37.5%[3] of the 24 restaurants examined showed "typical" oral streptococci still present on supposedly clean drinking glasses. The sanitary significance of these findings is obvious.

Summary. An improved technic for demonstrating the presence of oral streptococci on washed glasses is presented. The procedure involves primary enrichment of swab cultures in azide broth followed by inoculation onto sucrose-crystal violet-postassium tellurite agar. The simplicity of the technic, the ease of preparation of the media involved and the readily distinguishable colonies formed by *S. salivarius* on the selective agar substrate makes this procedure applicable to routine examination of restaurant ware. The data obtained by such routine examinations has public health significance which cannot be too strongly emphasized.

† Details will be reported more fully in another publication.

‡ Strain No. S44A received from Dr. James M. Sherman, Cornell, Ithaca, New York.

14170 P

Etiology of Renal Failure Following Crush Injuries.*

R. J. Bing.

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The work of Bywaters[1] has indicated that renal failure following crush injuries may be caused by the excretion of myoglobin. In a recent observation by the same author the injection of myoglobin into the acidified rabbit was seen to produce death from renal failure.[2] The present study was undertaken to examine the effect of dog's metmyoglobin on the renal function of the acidified dog and to compare its action with that of dog's hemoglobin and methemoglobin in the acidified and normal animal.

Methods. All experiments were performed on unanesthetized trained female dogs. Creatinine and mannitol clearances were used to measure glomerular filtration rate, and *p*-aminohippuric acid clearances for the determination of the effective renal plasma flow.[3] Alternatively, 60 to 80 cc of dog's blood which had been hemolyzed by freezing and thawing, from 4.1 to 7.8 g of crystalline dog's hemoglobin, or 5 g of metmyoglobin or myoglobin were infused into the leg vein of dogs acidified with 100 g of ammonium chloride *per os*. Crystalline dog hemoglobin and methemoglobin were prepared by the usual procedure using ammonium sulfate to salt out the proteins, a slight excess of potassium ferricyanide being used to oxydise hemoglobin to methemoglobin. Metmyoglobin and myoglobin were prepared from dog muscle using a slight modification of the method of Morgan.[4] In a series of control experiments acidosis alone was produced by feeding 100 g of ammonium chloride or by infusion of lactic acid varying in strength from 4 to 6%.

Results and Discussion. The infusion of hemolysed blood or crystalline hemoglobin into a series of 4 animals acidified with ammonium chloride produced no effect on the renal plasma flow and filtration rate during the period of infusion or in the following 3 days. In 3 experiments in which hemolysed blood or crystalline hemoglobin were infused into dogs with normal alkali reserve, similar negative results were obtained.

The effect of metmyoglobin on acidotic dogs was studied in 5 experiments. The infusion of this pigment produced no change in the renal clearances of creatinine or mannitol and *p*-aminohippuric acid during the 10 days of observation. No increase in blood urea occurred at any time. No pathological changes were present in these kidneys. These results differ from those obtained by Bywaters on the acidotic rabbit.[2] It is possible that this discrepancy represents differences in the reaction of the test animals.

The effect of methemoglobin infusion into animals acidified with ammonium chloride was observed in 12 experiments. In 2 of these the infusion failed to cause renal failure. In 10 instances, however, the onset of infusion was followed by a rapid decline in the glomerular filtration rate and the renal plasma flow. During the 3 days following the infusion the renal clearances fell to 5% of their normal control values. The blood pH remained below 7.2, and the blood urea rose to levels ranging from 140 and 200 mg %. The kidneys of these animals, which died or were sacrificed on the third day after the infusion, showed hydropic degeneration of the proximal convoluted tubules, cellular necrosis in the distal tubules, and some cast formation. Similar lesions have been observed by Bywaters and Dible in patients with crush syndrome.[5]

In 5 experiments dealing with the effect of acidosis produced by ammonium chloride alone, no fall in the clearances of creatinine or

* Aided by a grant from the Josiah Macy, Jr., Foundation.

1 Beall, D., Bywaters, E. G. L., Belsey, R. H. R., and Miles, J. A. R., *Brit. Med. J.*, 1941, **1**, 432.

2 Bywaters, E. G. L., *Brit. Med. J.*, 1942, **2**, 643.

3 Finkelstein, N., Aliminosa, L. M., and Smith, H. W., *Am. J. Phys.*, 1941, **133**, 276.

4 Morgan, V. E., *J. Biol. Chem.*, 1937, **112**, 557.

5 Bywaters, E. G. L., and Dible, J. H., *J. Path. Bact.*, 1942, **54**, 111.

p-aminohippuric acid were observed. However, when the acidosis was produced, by infusion of lactic acid (5 cases), a marked fall in all clearances was observed, coinciding with the appearance of methemoglobin in the urine. This observation makes it apparent that the renal failure was caused by the combined effect of acidosis and methemoglobin.

Summary. The infusion of crystalline methemoglobin into dogs acidified with ammonium chloride is followed by a fall in the effective renal plasma flow and the glomerular filtration rate of the dog. The dog may die in uremia on the third day following the infusion. Infusions of metmyoglobin and hemoglobin into acidified animals, and of methemoglobin and hemoglobin into normal animals, fail to depress renal function. The renal lesions of acidified dogs infused with methemoglobin resemble those observed in cases of crush syndrome and blackwater fever.

I want to express my gratitude to Louise Buchanan and Christine Waples for their assistance in carrying out these experiments, to Doctor Young for the preparation of the hemoglobin and myohemoglobin preparations, and to Doctor A. Cournand for his help in performing the blood chemistry.

14171

Excretion of Penicillin in Man.*

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Florey[1] has demonstrated that penicillin appears in the urine when administered to the experimental animal or to man. More recently Rammelkamp and Keefer[2] showed that 58% of penicillin administered by the intravenous route is excreted by the kidneys largely during the first hour after the injection. This rapid loss of penicillin in the urine is undesirable since frequent injections are required to maintain adequate concentrations in the blood during the treatment of infections. The fact that substances, such as diodrast, which are excreted by the renal tubules exhibit a similar rapidity of elimination suggested the possibility that penicillin is also excreted by the tubules in addition to being filtered through the glomeruli, and further that this portal of exit could be blocked and the elimination of penicillin decreased. The present study was undertaken, therefore, to determine the mechanism of excretion of penicillin.

Methods. Penicillin† was used in the form of the sodium salt dissolved in 0.85% sodium chloride in a concentration of 1000 Florey units per cubic centimeter. All injections were made with this standard solution.

In one subject a constant intravenous infusion of penicillin was administered for a period of 5½ hours. The flow was adjusted so that 9600 Florey units per hour were delivered. The urine collected 30 minutes after institution of the constant drip of penicillin was discarded, thereafter hourly collections of urine were made. From the second to the fourth hours diodrast was given at the rate of 0.3 cc per minute after a priming dose of 10 cc.

The elimination of penicillin was studied in 5 subjects after a single injection of 5000 Florey units and again 24 hours later after the simultaneous injection of 5000 Florey

* Supported by a grant from the Johnson Research Foundation, New Brunswick, N.J.

1 Florey, H. W., Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., and Jennings, M. A., *Lancet*, 1941, **2**, 177.

2 Rammelkamp, C. H., and Keefer, C. S., *J. Clin. Invest.*, 1943, **22**, 425.

† Penicillin was supplied through the courtesy of Dr. George A. Harrop, Squibb Institute for Medical Research, New Brunswick, N.J.

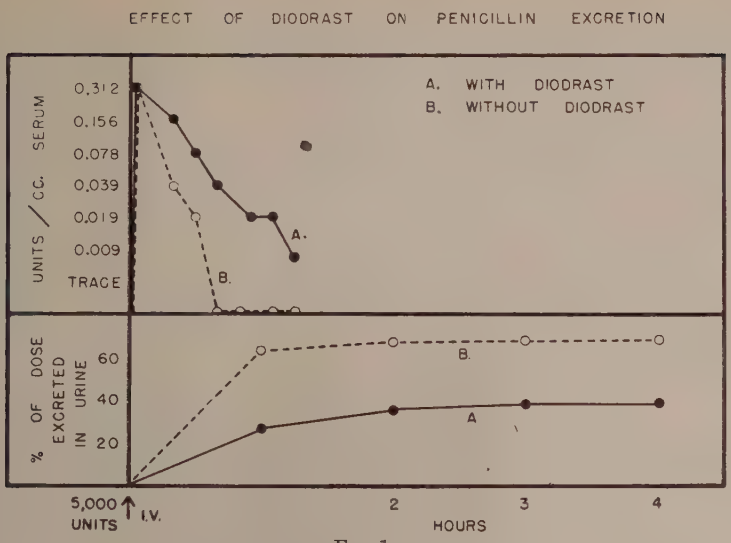

FIG. 1.

units and diodrast. On 2 occasions diodrast was given as a single injection of 30 cc just prior to the penicillin while in the remaining 3 instances 20 cc of diodrast was injected with the penicillin and 30 minutes later the administration of diodrast was repeated. Blood was withdrawn in sterile tubes at frequent intervals. Each subject ingested 400 cc of water every hour for 4 hours. Voided specimens of urine were collected in sterile containers at hourly intervals. The concentration of penicillin was then determined on each blood and urine sample using the method described previously.[3] No effect of diodrast upon bactericidal activity of penicillin was observed.

Results. When penicillin was administered to a normal subject by a constant intravenous drip delivering 9600 Florey units per hour, the excretion of the material by the kidneys was depressed by the addition of diodrast to the infusion. In this subject control periods of one hour both before and after the simultaneous injection of penicillin and diodrast showed excretion of 4530 and 3931 Florey units, respectively. During the 2-hour period of penicillin-diodrast administration, only 1087 Florey units were excreted each hour. This suggested that diodrast depressed the excretion of penicillin and, therefore, 5 additional subjects were studied after single injections of penicillin and penicillin-diodrast solutions.

In the 5 subjects who received 5000 Florey units and 24 hours later a second injection of 5000 Florey units and 30 to 40 cc of diodrast, the serum concentrations showed that the level of penicillin remained elevated to a greater height and for a longer period of time when diodrast was injected simultaneously. Fig. 1 shows the results obtained in Subject 6. In this instance, one minute after the injection of penicillin or penicillin-diodrast the serum contained 0.312 Florey unit per cubic centimeter. Penicillin disappeared from the circulating blood within 40 minutes after 5000 Florey units of penicillin, whereas the injection of penicillin-diodrast resulted in the maintenance of a significant level of penicillin in the serum for 75 minutes. In the remaining 4 subjects (Nos. 2, 3, 4, 5) the serum concentrations of penicillin were invariably higher and remained elevated for a longer period of time when diodrast was injected simultaneously with the penicillin.

The excretion of penicillin in the urine was greatly depressed by the injection of diodrast. As demonstrated in Fig. 1, 69% of the administered dose of penicillin was excreted in the urine within 4 hours while the total excretion

[3] Rammelkamp, C. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 95.

TABLE I.
Effect of Diodrast on Penicillin Excretion in Normal Subjects. Intravenous Injection of Penicillin in 5000 Florey Units.

Case	Diodrast, cc	% of penicillin excreted in urine per hour*			
		1	2	3	4
2	30	16	31	34	34
	0	40	47	48	48
3	30	32	37	39	39
	0	56	58	59	59
4	40†	19	24	25	26
	0	62	65	66	67
5	40†	17	24	25	25
	0	38	41	43	43
6	40†	27	35	36	36
	0	63	67	68	69

* Cumulative excretion.
† Initial injection of 20 cc of diodrast followed in 30 minutes by a second injection of 20 cc.

of penicillin when diodrast was given simultaneously amounted only to 36%. Most of the penicillin was excreted within the first hour after its injection on every occasion (Table I), and this was true both with and without the addition of diodrast. The average excretion of penicillin during the first hour in all subjects when diodrast was not given was 51.8% and after 4 hours was 57.2%. In contrast, when diodrast was given simultaneously, the average excretion during the first hour fell to 22.2%, and during the 4-hour period to 32%.

Discussion. Smith and his co-workers[4] have shown that there is mutual depression of the diodrast, hippuran, and phenol red clearances when these substances are given simultaneously, and more recently a similar relation between diodrast and other pyridone derivatives has been demonstrated.[5] When these substances are given simultaneously, there is a marked depression of all clearances but diodrast, which is lowered to only a slight extent. This phenomenon of mutual depression of tubular excretion is attributed to a competition for elimination through a common tubular mechanism. Since penicillin elimination is decreased in the presence of diodrast, it is probable that it is excreted by the tubules, perhaps largely by the same mechanism responsible for tubular excretion of diodrast and the other substances depressed by it. This may imply the presence of common chemical groups or common physicochemical properties. It also suggests the possibility that elimination of penicillin may be decreased during clinical use, thus maintaining adequate blood levels, by simultaneous administration of substances which block its excretion or that structure of penicillin may be altered in such a way as to produce this effect.

Of considerable interest is the fact that the total amount of penicillin excreted after diodrast-penicillin administration averaged only 32%, whereas 57.2% was excreted after the injection of penicillin alone. This suggests that penicillin is destroyed or inactivated within the body to a greater extent following diodrast administration and confirms the previous suggestion[2] that the degree of "inactivation" of penicillin is related to the time of exposure within the body.

Conclusion. The excretion of penicillin in man may be partially blocked by the simultaneous administration of diodrast.

4 Smith, H. W., Goldring, W., and Chasis, H., *J. Clin. Invest.*, 1938, **18**, 263.

5 Finkelstein, N., Aliminosa, L., and Smith, H. W., *Am. J. Physiol.*, 1941, **133**, 276.

14172 P

Induced Resistance to Penicillin of Cultures of Staphylococci, Pneumococci and Streptococci.

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It has been repeatedly shown that various infectious agents on exposure to certain substances acquire resistance to those substances. Abraham, Chain and collaborators[1] have rendered a culture of *Staphylococcus aureus* resistant to penicillin by growing it in broth containing increasing concentrations of penicillin. Rammelkamp and Maxon[2] have reported the development of resistance *in vitro* to penicillin by two strains of staphylococci. They also reported the recovery of resistant strains from 4 out of 14 patients with staphylococcal infections which were treated with penicillin.

It seemed of interest to determine if other organisms could likewise be rendered resistant to penicillin. Three strains of pneumococci, Types I, II, and III, one strain of *Streptococcus pyogenes*, C-203, and 3 strains of staphylococci were passed serially in broth containing the greatest amount of penicillin which would permit growth. Control cultures were passed in the same broth without penicillin. Subcultures were made at frequent (usually daily) intervals. Fuller details of this work will be reported elsewhere.[3] The development of resistance was shown by the ability of the cultures to grow in increasing concentrations of penicillin. At intervals comparative tests of the resistance of control and penicillin-passed cultures were made. The passages in penicillin broth extended over a period of 3 months and with all but 2 of the cultures 56 to 60 passages were made. These 2, both staphylococcus cultures, had only 28 passages each and acquired a resistance respectively 1000 and 4000 times greater than that of the control cultures. The third staphylococcus culture which had 60 passages acquired a 6000-fold increase in resistance. The Type III pneumococcus and the *Streptococcus pyogenes* acquired a 30-fold increase in resistance, while the Type I and Type II pneumococci showed only a 6-fold increase.

Cultures which have become resistant to penicillin, unlike those resistant to sulfonamides, show marked decrease in virulence for mice. Five of the 7 cultures passed in penicillin broth lost virulence to a marked degree, the 2 which showed only slight loss were the Type I and Type II pneumococcus cultures which had acquired only 6-fold resistance *in vitro*. The other cultures, with increase in resistance varying from 30- to 6000-fold, showed equal and almost complete loss in virulence. Cultures which, before passage, killed regularly in a dilution of 10^{-7} after passage killed only irregularly in a dilution of 10^{-1}. This loss of virulence, once acquired, appears to be a permanent characteristic of the organism. The Type III pneumococcus resistant culture was subjected to 9 serial passages in mice and 32 rapid transfers in blood broth. Neither procedure increased virulence or decreased resistance to penicillin *in vitro*. After passages in penicillin broth were stopped all the cultures were stored in the icebox with only occasional transfers to fresh tubes of broth. After 2 months storage there was no change in either resistance *in vitro* or virulence. The 2 least resistant staphylococcus cultures were not tested. It was noticed, however, that the penicillin resistant strains grew more quickly on transfer to fresh broth, and colonies on blood agar grew more luxuriantly than at the time passage in penicillin broth was stopped.

[1] Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., Jennings, M. A., and Florey, H. W., *Lancet*, 1941, **2**, 177.

[2] Rammelkamp, D. H., and Maxon, T., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 95.

[3] Rake, G., McKee, C. M., Hamre, D. M., and Houck, C. L., to be published.

Increase in resistance to penicillin and loss of virulence have been accompanied by other changes. The rate of growth was retarded and the type of colony became more variable, especially in those cultures showing the greater resistance. Bile solubility of the pneumococcus cultures and type specificity, as determined by the *Quellung* method, were unchanged. Carbohydrate fermentation was the same with resistant and control cultures although it occurred more slowly with the former.

Using the staphylococcus Smith culture, we have been unable to demonstrate acquisition of resistance to penicillin by *in vivo* methods. Twenty serial passages through mice treated with penicillin increased resistance only proportionally to increase in virulence.

Cultures rendered resistant to penicillin were, in our hands, unchanged in their resistance to the sulfonamides, and also to other antibiotic substances, namely, gramicidin, gliotoxin, and aspergillic acid.

Summary. Three cultures of staphylococci, one of *Streptococcus pyogenes* and 3 of pneumococci, Types I, II, and III, showed wide differences in degree of resistance after cultivation in presence of penicillin.

Loss in virulence for mice was associated with increase in resistance. Serial passage of one such strain through mice failed to restore virulence.

Passage *in vivo* in the presence of penicillin failed to increase specific resistance of a strain of *Staphylococcus aureus*.

14173

Calcium Deficiency and Gastric Lesions in the Rat.

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Gastric lesions resulting from dietary deficiencies have been described by a number of investigators. As to their cause, almost as many opinions have been expressed. Most of the observations have been made with diets which were deficient in a number of factors.

We have satisfied ourselves that in case of the multiple deficiency diets usually employed the addition of either protein, thiamin, or riboflavin may affect the frequency of lesions or their distribution over the mucosal area. Three histologically different types are found in the 3 anatomically distinguished parts (rumen, antrum, and fundus) of the rat's stomach. Berg[1] has described the histological findings with typical multiple deficiency diets and has given references to previous investigators. The distribution of the lesions is not haphazard, but only partial success has been achieved so far in correlating a single deficiency with a given type of lesion.

In the meantime, it has appeared that another deficiency, namely that of calcium, must be considered. From experiments partly carried out for other purposes, it became apparent that diets adequate for excellent growth in all respects will lead to a high incidence of predominantly antral lesions if calcium is omitted from the salt mixture. These lesions had the same typical appearance as the antral lesions described by Berg: round hyperplastic areas with a central depression representing a defect in the mucosa.

To illustrate this, two series of experiments will be cited. All animals were on the experimental regime for 28 days. Different groups had initial ages of 4, 7, and 10 weeks. The record is presented as the mean count of antrum lesions per rat in each group. The count is based on gross inspection of the stretched out stomach wall. The antral mucosa may contain lymph nodules indistinguishable grossly from the lesions which appear as roundish slightly raised areas with a depressed

[1] Berg, B. N., *Am. J. Path.*, 1942, **18**, 49.

center. A long series of histological controls has shown that lymph nodules are not likely to average as high as one per rat even in small groups. In 2 out of over a hundred instances as many as 3 nodules were found in a single rat, while about half of the animals showed no nodules. A count of less than one therefore stands for a normal antral mucosa.

The basal diet of Series 1 contained: Heated egg albumen, 20%; modified Wesson salt mixture, 1.2%; cottonseed oil (containing carotin), 2%; rice bran extract, 5%; P (as KH_2PO_4), 0.4%; cane sugar to make 100%. This was the experimental diet in Series 1. The control diet had 0.6% of Ca added as $CaCO_3$.

Omitting the account of intermediate levels which gave intermediate results, the first series comprised 70 low Ca animals and 65 controls. In groups of 4 or 5 animals of each sex, they were spread over the time from June, 1941, to October, 1942, with no noticeable differences in the summer and winter months. On certain other diets, such as those mentioned by Berg, very marked seasonal differences are obvious.

The groups of 8 to 10 animals showed a mean count of 10 to 18 lesions per rat, averaging 15 for the total of 70 animals. A small number of fundic and rumen lesions were a constant accompaniment of antral lesions on such diets, while with many multiple deficiency diets all types occurred freely. Not infrequently the same stomach showed marked lesions in rumen, antrum, and fundus.

The mean count for the 65 control stomachs was 0.83 per rat with no group being responsible for more than the average except one which contained a rat with 16 lesions. This sporadic occurrence of numerous gastric lesions in normal control groups has also been encountered in other series of experiments. Stock diet animals also show abnormal gastric mucosæ to the extent of about one in a hundred.

Growth and food intake on Ca deficient diets may remain approximately normal for 7 to 10 days. After this, both fall off sharply. The protein and B complex allowances in the diets mentioned above are quite adequate with normal food intakes. It might be held that in such an experiment the effective cause need not be Ca directly, but rather a deficiency induced by lowered intakes of other factors. The second series aimed at minimizing the chances of such an occurrence.

In the second series, the protein was raised to 26.7%. The rice bran extract which as a source of B complex is relatively deficient in riboflavin was supplemented by adding 100 γ of riboflavin per 100 g of diet. In Series 1, the egg albumen supplied considerable riboflavin. An additional source of B complex (Harris yeast extract) was used to insure against borderline values of the less well worked out or unidentified B factors. The phosphate added to the diet was adjusted for the P content of the casein. The constituents common to the 4 diets used were as follows: Casein, 26.7%; cottonseed oil and carotin, 2.0%; modified Wesson salts, 1.2%; KH_2PO_4, 0.66%; rice bran extract, 5%; riboflavin, 100 γ; cerelose to 100%. The variables and the antrum lesion counts are given below:

	Diet 1015	Diet 1016	Diet 1018	Diet 1017
Yeast extract	0.5%	0.5%	2.0%	2.0%
$CaCO_3$	1.49%	0	1.49%	0
No. of rats	8	9	6	15
Lesion count	0	24	0	18

Six rats on diet 1018 were pair-fed to 6 rats on 1017. With the food intake on the adequate diet reduced (maximally about 50%) to that of the calcium deficient one, the gastric mucosa remained perfectly normal. While in the experiments of Series 1 very few lesions in either rumen or fundus were seen, Series 2 showed none. The lesions were exclusively antral.

Discussion. A comparison of Series 1 and 2 appears to give more information than merely corroborating the indication that it is a calcium deficiency *per se* which produces the result. Since on no other diets except those of Series 2 have we ever obtained antrum lesions uncomplicated by fundus or rumen lesions, the conclusion seems warranted that reduced food intake can induce complications unless secondary deficiencies are guarded against.

From the experiments given here it is clear that vitamin D deficiency plays no primary role in causation of the lesions. With no other change in the diet, marked lesions or complete

prevention are the result of omission of calcium from or addition of calcium to the diet, respectively. Therefore, since there is complete prevention, in the absence of vitamin D, calcium deficiency *per se* must be regarded as the causative factor. The situation is analogous to that of phosphorus deficiency in experimental rat rickets (Zucker *et al.*,[2] pp. 147-8). Vitamin D cannot fully compensate for very low supplies of the inorganic essential substances (calcium or phosphorus). Only with moderate deficiencies is it possible for vitamin D to improve the net absorption sufficiently to effect prevention. The preventive role of D in gastric lesions produced by moderate calcium deficiency together with other findings in calcium deficiency will be discussed in another report.

Preliminary experiments indicate that the results of Schiödt,[3] who apparently produced exclusively fundus lesions, can be duplicated. No one else has reported such results and we have not seen them until recently. Here the deficiency seems to be one of the B factors as indicated by Schiödt. Further details are now under investigation.

Conclusion. Antral gastric lesions in the rat are produced by calcium deficiency.

[2] Zucker, T. F., Hall, L., and Young, M., *J. Nutrition*, 1941, **22**, 139.

[3] Schiödt, E., *Acta Med. Scand.*, 1934-35, **84**, 456.

14174

Complement Fixation Test with Sera of Animals Immunized with Rabies Virus.

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Recently, a reliable technic for complement fixation for diagnosis of rabies was devised by Casals and Palacios.[1] The procedure for preparing antigen is, however, complicated and it seemed desirable to develop a simpler technic for complement fixation which might find application in routine laboratory diagnosis. At first, specific and sharp results were obtained by following the technic developed by Howitt;[2] subsequent experiments showed, however, that further simplification was possible and the following technic was finally evolved, which offers a practical procedure for routine diagnosis.

Antigen. One to 2 g of rabies-infected brain are minced in a Petri dish, spread in a thin layer, and dried in a desiccator over sulphuric acid, at 37°C for 24 hours.* The dried material is scraped off and stored in rubber-stoppered tubes in the ice box. Material thus preserved keeps at least 9 months. For the preparation of antigen, the dried material is ground in a mortar, taken up in neutral physiological saline and added gradually to give a final concentration of 2%. The suspension is centrifuged for 15 minutes at 3000 r.p.m. The supernatant fluid serves as the antigen. This type of antigen can be prepared from the brains of rabbits, guinea pigs, mice, and dogs, infected intracerebrally or peripherally with mouse passage fixed virus, virus from tissue cultures,[3,4] egg passage virus,[5] or with a local strain of street virus. Antigens prepared from brains of guinea pigs and mice infected with equine encephalitis

[1] Casals, J., and Palacios, R., *Science*, 1941, **93**, 162; *J. Exp. Med.*, 1941, **74**, 409.

[2] Howitt, B. F., *J. Immunol.*, 1937, **33**, 235.

* The preliminary drying is important. Antigens prepared from fresh undried material were found anticomplementary.

[3] Webster, L. T., and Clow, A. D., *Science*, 1936, **84**, 487; *J. Exp. Med.*, 1937, **66**, 125.

[4] Bernkopf, H., and Kligler, I. J., *Brit. J. Exp. Path.*, 1937, **18**, 481.

[5] Kligler, I. J., and Bernkopf, H., *Nature*, 1939, **143**, 899; Bernkopf, H., and Kligler, I. J., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 332.

TABLE I.
Complement Fixation with Brain Antigens from Animals Infected with Rabies and Other Viruses.

Guinea pig antisera obtained by immunization with	Antigens						
	Normal brains	Murine typhus	Equine encephalitis virus	Street virus	Egg passage virus	Tissue culture virus	Brain passage fixed virus
Brain passage fixed virus	—	—	—	+	+	+	+
Tissue culture virus	—	—	n	n	+	n	+
Street virus	—	—	—	+	n	n	+
Equine encephalitis virus	—	—	+	—	n	n	—
Control, normal guinea pig sera	—	—	—	—	—	—	—

+ fixation.
— no fixation.
n not tested.

virus (eastern strain), brains of guinea pigs infected with murine typhus, and normal brains of mice, guinea pigs, rabbits, and dogs served as negative controls.

Sera. Sera of immunized guinea pigs were employed as source of antibody. The animals received a 10% suspension of homologous rabies brain vaccine inactivated with 1% formalin. Three courses of immunization are administered, each consisting of 4 injections of 5 cc, given at 4-day intervals. One group of guinea pigs was immunized with living culture virus and one with a formalin-inactivated strain of street virus. Sera of guinea pigs vaccinated with the virus of equine encephalitis and normal guinea pig sera were used as controls. All sera was inactivated at 56°C for 30 minutes.

Complement fixation test. The test was set up according to Eagle's technic[6] for the Wassermann reaction, using a total volume of 1 cc and 2 units of amboceptor. Our antigens, even in the highest concentration, did not exhibit anticomplementary reactions. Fixation was carried out for 16-18 hours in the ice box, followed by 30 minutes at room temperature. Water bath fixation at 37°C was not sufficiently sensitive. After addition of the hemolytic system, the mixture was incubated for 30 minutes in a 37°C water bath and the results were read as soon as tubes containing antigen and serum controls became clear.

Results. Typical results are summarized in Table I. Anti-rabies immune sera produced by the procedure outlined gave specific results and failed to react with brain antigens of animals infected with typhus or equine encephalitis or of normal brains.† Normal sera were always negative.

Comparative experiments with our strains of street and fixed virus yielded the following results:

Antisera produced by immunization with street virus (local strain), fixed complement in lower titers than did sera of animals immunized with fixed virus, when either fixed virus or street virus brain was employed as test antigen. The latter sera reacted up to a dilution of 1:400 with standard antigens prepared from brain of mice infected with fixed virus. When brains of dogs infected with our strain of street virus were employed as antigen, the titers of these sera were lower. Thus, sera with a maximum titer of 1:125 failed to react with brains of dogs infected with street virus; positive results were obtained with this antigen only when sera of a titer of at least 1:250 were employed. This may perhaps be due to a lower concentration of antigen in dog brain than in mouse brain. When different parts of dog brains were tested for their antigen content, considerable differences were observed; the highest titer was always found with medulla which gave reactions when diluted 1:16, while cortex, midbrain, and cerebellum

[6] Eagle, H., *The Laboratory Diagnosis of Syphilis*, 1937, C. V. Mosby Co., St. Louis.

† Satisfactory antigens were obtained from partly decomposed brains of animals which had died of rabies.

gave consistently lower titers.

The technic used for the preparation of rabies antigen proved unsuitable for the preparation of antigens from brains of animals infected with equine encephalitis virus. It appears that simple desiccation is detrimental to this antigen. Frozen brains dried in a vacuum yielded antigens which reacted with anti-equine encephalitis sera but exhibited marked anti-complementary properties.

The rate of appearance and the titer of neutralizing and complement fixing antibodies respectively, were observed during a period of 4 months in a group of 5 guinea pigs which had received one course of immunization against rabies. The complement fixing antibodies reached a maximum titer about a week after the last injection, decreased to ⅛ of the maximum titer after one month and disappeared completely after 4 months. The neutralizing antibodies, however, reached a maximum titer after about one month and did not show a decrease during the subsequent period of observation. The results indicated a lack of correlation of complement fixing with virus neutralizing antibodies in rabies, and are in accord with the findings of Casals and Palacios.

14175

Effect of Growth Hormone on Glycosuria of Fed Partially Depancreatized Rats.*

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Although it has been well established that the secretions of the anterior pituitary profoundly influence carbohydrate metabolism, it has not been shown as yet which hormone or hormones are involved in this relationship. This problem has recently been comprehensively reviewed by Houssay.[1] Heretofore investigators have used whole pituitary extracts or hormone preparations only partially purified. Recently much progress has been made in the isolation of the anterior pituitary hormones. We have available at present the following hormones in a state which approaches physiological purity: growth, lactogenic, interstitial cell stimulating, and adrenocorticotropic.

A study is being carried out to determine which of the hormones of the anterior pituitary will produce glycosuria in the partially depancreatized rat. The data presented here have to do only with the effect of growth hormone upon the glucose excretion of such an animal.

Methods. The test animal used in these experiments is a potentially diabetic male rat in which a partial pancreatectomy had been performed 5 to 6 months previously, when the animal was 4 to 5 weeks of age. The rats were fed a standard diet except for the 24-hour periods in which the glycosuria was measured. During this period, the animals were placed in individual metabolic cages, and given 75 cc of a 20% sucrose solution as their only food and drink. All animals but one operated and one normal drank the total amount. Under these conditions it was found that the partially depancreatized rat excretes from 0.1 to 4.0 g of glucose in 24 hours. The unoperated rat of the same age excretes as a rule less than 0.1 g of glucose during 24 hours.

The growth hormone preparation used was a "cysteine treated globulin fraction," prepared as reported recently,[2] which had a potency of approximately 23 growth hormone units per milligram (hypophysectomized rat

* Aided by grants from the Board of Research of the University of California and the National Research Council Committee on Research in Endocrinology.

1 Houssay, B. A., *Endocrinology*, 1942, **30**, 884.

2 Marx, W., Simpson, M. E., and Evans, H. M., *J. Biol. Chem.*, 1943, **147**, 77.

TABLE I.

Test animals	Total dose growth hormone, mg	Urinary glucose in 24 hours in individual rats		
		Before injection, g	On 3rd day of injection, g	Increase, g
Partially depancreatized rats	15.0	3.74	4.42	0.68
		2.20	4.75	2.55
		1.85	3.35	1.50
		1.69	4.85	3.16
		0.64	4.00	3.36
		0.52	1.30	0.78
		0.33	1.40	1.07
		0.23	0.95	0.72
		0.17	0.28	0.11
		0.12	0.56	0.44
		0.11	0.90	0.79
		0.10	0.75	0.65
		0.06	0.52	0.46
	7.5	2.86	3.10	0.24
		0.18	1.24	1.06
		0.18	0.27	0.09
		0.12	0.09	—0.03
		0.04	0.06	0.02
	1.5	0.70	0.94	0.24
		0.70	0.53	—0.17
		0.10	0.25	0.15
		0.08	0.11	0.03
		0.06	0.09	0.03
Normal rats	15.0	0.14	0.08	—0.06
		0.06	0.08	0.02
		0.03	0.03	0.00
		0.02	0.06	0.04

units). When this preparation was injected into hypophysectomized rats at a total dose of 5.0 mg (4-day test), no stimulation of ovaries or adrenals, and only a trace of thyrotropic activity was detectable. When injected subcutaneously into immature pigeons at a total dose of 10.0 mg (4-day test) no lactogenic hormone was demonstrable.

A control measurement of the glucose excretion per 24 hours was taken a few days before the injections of growth hormone were started. The hormone was given once daily for 3 days at levels varying from 1.5 to 15.0 mg total dose. During the last 24 hours of the 3-day injection period the glucose excretion was again measured.

Results. The glucose excretion before and after growth hormone administration is reported in columns 3 and 4 of Table I. A striking increase in the amount of glucose excreted occurred in most of the rats after injection with growth hormone (column 5). There was no uniformity in the amount of glycosuria before injection, nor in the response of the rats to a given dose of growth hormone. This is interpreted as being due chiefly to the fact that the amount of pancreatic tissue removed at operation varied considerably.

Conclusion. It has been shown that a purified growth hormone preparation of the anterior pituitary which is practically free of lactogenic, adrenocorticotropic, thyrotropic, and gonadotropic hormones, produces a marked increase in glucose excretion in the sucrose-fed partially depancreatized rat.

14176

Hypoprothrombinemia After Salicylate Administration in Man and Rabbits.

S. Rapoport, Mary Wing, and George Martin Guest. (Introduced by A. A. Weech.)

From the Children's Hospital Research Foundation and the Department of Pediatrics, College of Medicine, University of Cincinnati, Cincinnati.

During investigations on the effects of large doses of salicylates on the electrolyte structure of the blood, to be reported elsewhere, it was noted that a number of salicylate-intoxicated rabbits died of pericardial hemorrhage following heart puncture, and that their blood sometimes required a long time to clot. Consequently, studies of the prothrombin content of such bloods were undertaken.

Recently Link and his associates reported the finding of hypoprothrombinemia in rats fed salicylates.[1] Earlier, they had found that coumarins induced hypoprothrombinemia in various species of animals,[2] a finding that gains interest in view of the chemical relationship between the salicylates and the coumarins.

Methods. As a measure of prothrombin, the clotting time of oxalate-plasma recalcified in the presence of an excess of thromboplastin was determined according to the method of Fullerton[3] slightly modified, using the venom of the Russel-viper as the thromboplastic agent. By this method the average prothrombin time of normal human plasma, with its standard deviation, was found to be 19.7 ± 1.8 seconds; of dog plasma, 11.3 ± 1.2 seconds; and of rabbit plasma, 11.8 ± 1.2 seconds. Studies were made on bloods of dogs and rabbits given large doses of methyl salicylate, and bloods of rheumatic fever patients (children) who were treated with salicylates.

Results. The dogs showed no change in prothrombin time following intramuscular injections of methyl salicylate in doses, 0.6 cc per kg, which gave rise to signs of severe intoxication (hyperpnea, etc.) and profound disturbances of the plasma electrolytes.

In rabbits, severe degrees of hypoprothrombinemia were found repeatedly at around 48 hours after the subcutaneous injection of 0.5 cc of methyl salicylate per kg. Several rabbits, however, died within a lesser time without showing such changes. Fig. 1 illustrates typical observations made on 2 rabbits exhibiting severe degrees of hypoprothrombinemia.

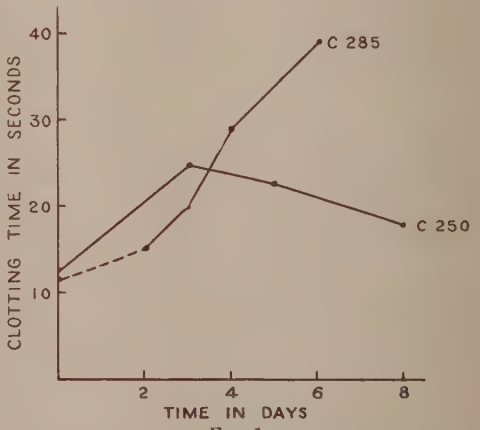

Fig. 1.

Prothrombin time of the blood plasma of 2 rabbits, C 250 and C 285, determined at varying intervals after the subcutaneous injection of 0.5 cc of methyl salicylate per kg.

Studies were made on 15 rheumatic patients, 6 to 14 years of age, during periods of salicylate medication ranging from 2 to 21 days. Thirteen received sodium salicylate and 2 received acetylsalicylic acid, in amounts varying from 1.5 to 8 g daily. Shifts in plasma electrolytes found in these patients will be reported elsewhere; but, suffice to state here, there was no apparent relationship between the chemical changes and changes in the prothrombin time of the blood. Manifestations of salicylism, such as vomiting, earache, diaphoresis, and/or marked hyperpnea, appeared

[1] Link, K. P., Overman, R. S., Sullivan, W. R., Huebner, C. F., and Scheel, L. D., *J. Biol. Chem.*, 1943, **147**, 463.

[2] Stahmann, M. A., Huebner, C. F., and Link, K. P., *J. Biol. Chem.*, 1941, **138**, 513.

[3] Fullerton, M. W., *Lancet*, 1940, **2**, 195.

TABLE I.
Prothrombin in the Blood Plasma of Rheumatic Patients Receiving Salicylate Medication.

Name	Age, yrs	Dosage, g per day	Time, relative to medication	Prothrombin time, sec	Prothrombin % of normal
A.H.	9	*0	Before	20.2	95
		4	2 days	24.7	65
		4	4 "	22.9	80
		0	1 " after	22.1	85
C.B.	13	*0	Before	20.1	95
		6	2 days	26.6	50
		6	4 "	25.5	55
		6	6 "	26.8	50
		6	8 "	25.8	55
		0	1 " after	23.2	80
H.M.	14	*0	Before	21.0	90
		6	4 days	23.9	70
		6	7 "	23.9	70
		0	5 " after	20.2	95
M.G.	9	*0	Before	20.8	90
		6	4 days	31.1	45
F.F.	12	*0	Before	22.0	85
		8	4 days	22.9	80
		8	21 "	24.9	65
V.P.	12	*0	Before	16.2	—
		6	5 days	24.1	70
		6	8 "	27.5	50
		0	5 " after	19.6	100
		6	5 "	24.8	70
H.V.	12	*0	Before	19.4	100
		6	9 days	21.4	90
L.O.	14	†8	3 "	24.8	65
		8	8 "	35.0	35
		8	9 "	30.2	45
		0	30 " after	19.1	100
		6	4 "	26.9	50

*Sodium salicylate.
†Acetylsalicylic acid.

in three of these patients. Table I presents data on 8 of these patients in whom significant prolongations of prothrombin time were found. The prothrombin values are listed both in seconds clotting time and in percentage of normal, the latter derived from the dilution curve of normal plasma according to Quick.[4]

The results indicate clearly that therapeutic doses of salicylates can lead to a marked prolongation of the prothrombin time of the blood. The evaluation of the significance of such changes awaits further study. It is conceivable that the hypoprothrombinemia reflects a certain degree of liver damage. A question also arises whether the hemorrhagic tendency occasionally present in rheumatic fever may complicate salicylate therapy or sometimes may be a concomitant effect of such therapy. The possibility of counteracting the effect of salicylate on prothrombin by the administration of vitamin K should be investigated.

[4] Quick, A. J., *Am. J. Phys.*, 1937, **118**, 260.

14177

Effects of Mononitroparaffins and Related Compounds on Blood Pressure and Respiration of Rabbits.

WILLARD MACHLE AND EUGENE W. SCOTT. (Introduced by W. Deichmann.)

From the Kettering Laboratory of Applied Physiology, College of Medicine, University of Cincinnati, Cincinnati, Ohio.

In the course of more elaborate study of the toxicity of mononitroparaffins and related compounds from the standpoint of industrial hygiene,[1] each substance was also examined for its effect on the blood pressure and respiration of rabbits. The first 6 members of the mononitroparaffin series, *i.e.*, nitromethane, nitroethane, 1- and 2-nitropropane, 1- and 2-nitrobutane, and 2-nitro-2-methyl-propanol, 2-nitro-2-methyl-,3-propanediol, tris-(hydroxymethyl) nitromethane, 2-amino-2-methyl-1-propanol, 2-amino-2-methyl-1,3-propanediol and tris-(hydroxymethyl)methylamine (the last three as hydrochlorides) failed to show any significant effect on either blood pressure or respiration. The same results were obtained in cats with the 6 nitroparaffins by W. Deichmann of this laboratory.

[1] Machle, W., Scott, E. W., and Treon, J., *J. Ind. Hyg. and Toxicol.*, 1940, **22**, 315.

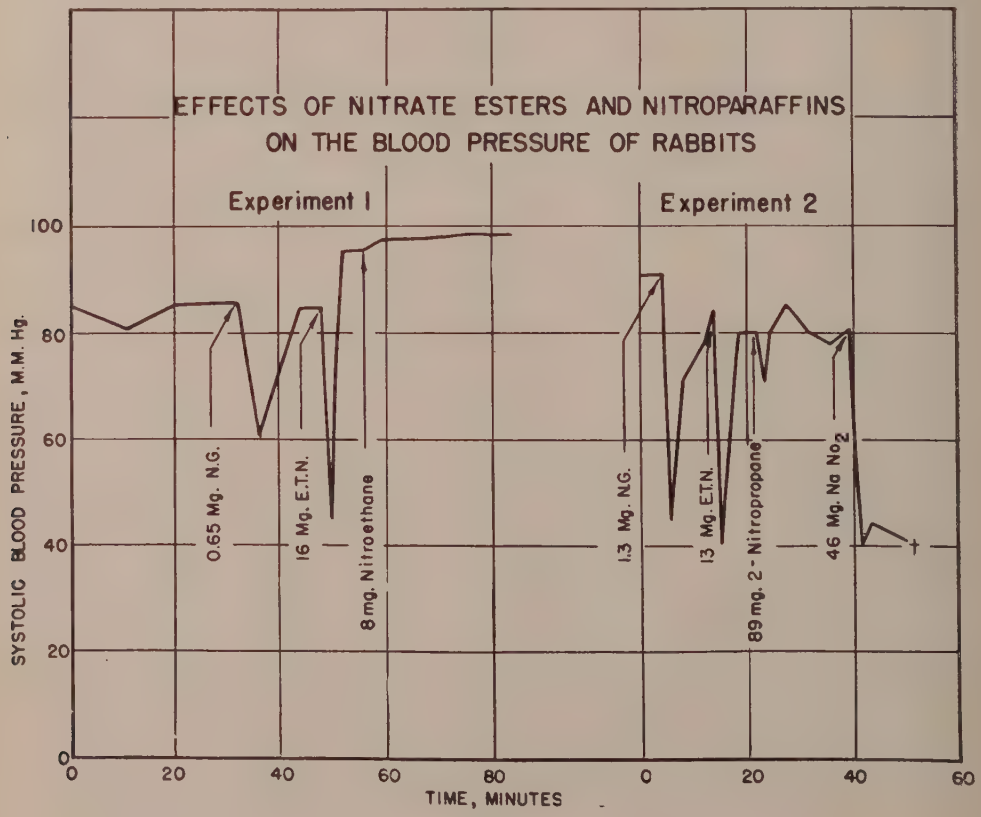

FIG. 1.

In addition, the effects of nitroethane and 2-nitropropane on the blood pressure of rabbits were compared with those induced by glycerol trinitrate, erythrityl tetranitrate and sodium nitrite. The results are shown in Fig. 1. The nitrates and sodium nitrite had very much more effect in much smaller doses than did the nitroparaffins.

14178

Occlusion of the External Pancreatic Secretion in Man.

ALEXANDER BRUNSCHWIG AND J. GARROTT ALLEN.
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Recent experience has shown that survival for at least many months is possible in man following apparent occlusion of the external pancreatic secretion. This situation obtains after pancreatoduodenectomy is performed for carcinoma of the head of the pancreas or ampulla of Vater. While an anatomic "cure" of such lesions might now be envisaged, it is not known if man can survive *indefinitely* without external pancreatic secretion. Data bearing on this question can only be obtained from a study of operated patients to observe evidence of impaired physiologic activity which itself may not permit of indefinite survival. In this connection it may be pointed out that where only a portion of the head of the pancreas and a portion of the duodenum have been resected, the patients are not suitable subjects for such studies since there is considerable variation in the pancreatic duct system and accessory ducts, anastomosing with the main pancreatic ducts, may enter the first or the terminal segments of duodenum.

The data recorded below were obtained upon 3 male patients, all subjected to a one-stage resection of the head and neck of pancreas, lower 4 to 6 cm of stomach, entire duodenum, and first 2 to 4 cm of jejunum (Fig. 1). Continuity of the alimentary tract was re-established by choledochojejunostomy, and gastro-jejunostomy. Two patients, Fl. and H, had large carcinomas of the head of the pancreas. Patient W. had a large carcinoma of the ampulla of Vater extending upward in the common bile duct. Postoperatively, none exhibited pancreatic or biliary fistulæ.

Patients Fl. and H. survived operation for 5½ and 5 months respectively dying of carcinomatosis. The latter condition was the cause of general deterioration which became most pronounced during the last 2½ and 2 months respectively. Patient W. remains well, has gained 25 pounds in weight and is engaged in physical labor 11 months after operation.

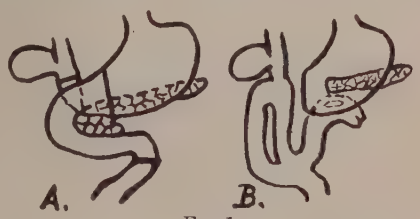

FIG. 1.

Diagram of one-stage pancreatoduodenectomy for excision of lower portion of stomach, entire duodenum and head of pancreas for carcinoma of the latter. A, showing levels of transection of dilated common bile duct, in lower portion of stomach, and in upper portion of jejunum. B, showing reconstitution of continuity of upper alimentary tract by gastrojejunostomy, choledochojejunostomy, and jejunostomy. The transected neck of pancreas is closed by mass ligature and sutures, thus resulting in occlusion of external pancreatic secretion.

Character of Stool. Patients Fl. and H. both had one or 2 daily large, bulky, soft, light tan or clay-colored foul stools following operation and during their survival period. Actual steatorrhea was present for a period in patient Fl., who had as many as 6 stools a day. Patient W. had steatorrhea prior to operation, but afterward, when bile alone was returned to the alimentary tract, the stools were normal.

TABLE I.
Blood Chemistry in 3 Patients with Occluded Pancreatic Ducts.

Patient	Plasma proteins, g %		N.P.N., mg %		Fasting dextrose, mg %		Blood amylase, Somogyi units	
	Pre-op.	Post-op.	Pre-op.	Post-op.	Pre-op.	Post-op.	Pre-op.	Post-op.
Fl.	6.57	6.18 (14 wks)	20	18.6 (14 wks)	111	92 (6 days)	—	31 (14 wks)
H.	6.34	6.06 (12 wks)	16	23 (4 wks)	81	65 (12 wks)	45	21 (8 wks)
W.	6.25	6.00 (13 wks)	32	30 (13 wks)	—	88 (13 wks)	266	105 (12 wks)

TABLE II.
Fat Absorption in 3 Patients with Excised Head of Pancreas and Duodenum Occluding Pancreatic Secretion.

Patient	Post-operative weeks	Fat intake g in days	Fat in stools g	% fat absorbed
Fl.	14	280 5	248	8.8
		219 3 (+ pancreatin)	142	35
H.	5	344 8	330	4
W.	12	450 8	37	91.6

TABLE III.
Blood Lipids and Cholesterol in 3 Patients with Occluded Pancreatic Secretion.

Patient	Time post-operative, weeks	Total lipids, mg %	Total cholesterol, mg %	Free cholesterol, mg %
Fl.	11	963	137.7	93.6
	16	630	42.4	27
	20	573	100	82.4
H.	3	1066	151.5	217.2
	6	577	54.5	36
	18	653	92.5	80
W.	7	576	84	48.4
	35	785	92	77.5

Meat Digestion. Raw ground beef was fed to each patient, 2 pounds, during a 24-hour period and subsequent stools inspected for undigested fragments. These were present in small quantities in patients Fl. and H., not in patient W.

Blood Chemistry. The observations on blood chemistry are summarized in Table I. No abnormalities are noted except for an elevated blood amylase in patient W preoperatively which became normal 3 months after operation.

Fat Absorption. For these studies the patients were afforded a uniform diet including 60 cc of olive oil, 12.5 g of butter fat, and 1.8 g of fat in milk a day. A capsule of carmine was administered and passage of carmine stools marked the beginning of a period during which all stools were collected; 5 to 8 days later a similar capsule was given and the appearance of the carmine in the stools marked the end of the period. The stools were emulsified in a mechanical mixer, extracted with alcohol-ether and petroleum ether, and the recovered fat weighed. The results of these studies are summarized in Table II. The 2 patients with fatty stools apparently absorbed only 8.8 and 4% respectively of fat ingested, whereas the patient with normal stools absorbed 91.6%. The increased

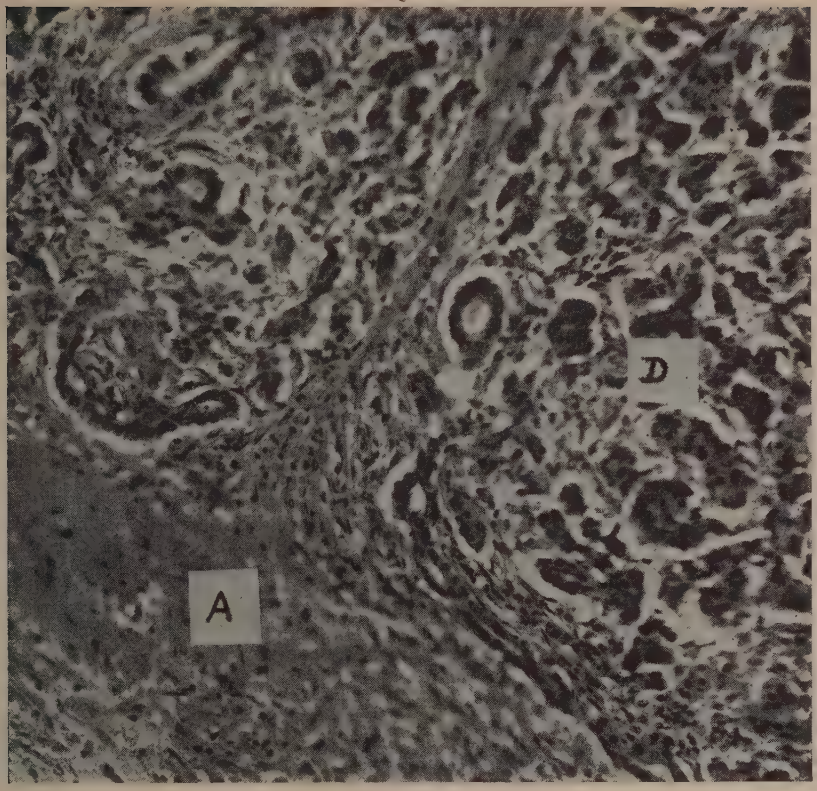

FIG. 2.

Photomicrographs × 200 of the remaining pancreas obtained at necropsy from patient Fl., 5 months after excision of the head of the pancreas and duodenum (occlusion of external pancreatic secretion). A. Fibrosis. D. Persisting acinar tissue, the cells of which are shrunken. Interacinar edema and fibrosis.

absorption of fat when pancreatin was administered is shown in patient Fl. Whipple and Bauman[1] observed 97% fat absorption in 2 patients from whom part of the duodenum and part of the head of the pancreas were resected, but in one instance there was considerable variation from 33% to 98% from day to day. In a third patient in whom the entire duodenum and head of pancreas were removed fat absorption varied daily from 0 to 92%.

Blood Cholesterol and Lipids. Results of these determinations are summarized in Table III. Some variation is observed, but the levels obtained a number of weeks after operation appear to be uniformly within the normal range. No significant changes are apparent. If the normal blood cholesterol is considered to be 100 to 300 mg % the results here obtained are also within normal ranges (with possible non-significant low readings observed in the second determinations in patients Fl. and H.).

Blood Cell Counts. No significance may be attached to the erythrocyte counts in patients Fl. and H., since secondary anemia developed in each case as carcinomatosis progressed; transfusions were given. In patient W. who has remained well the erythrocytes were 4.7 million, leucocytes 5,000, and Hb. 15 g %, 4 months after operation.

Necropsy Findings (Department of

1 Whipple, A. O., and Bauman, L., *Am. J. M. Sc.*, 1941, **201**, 629.

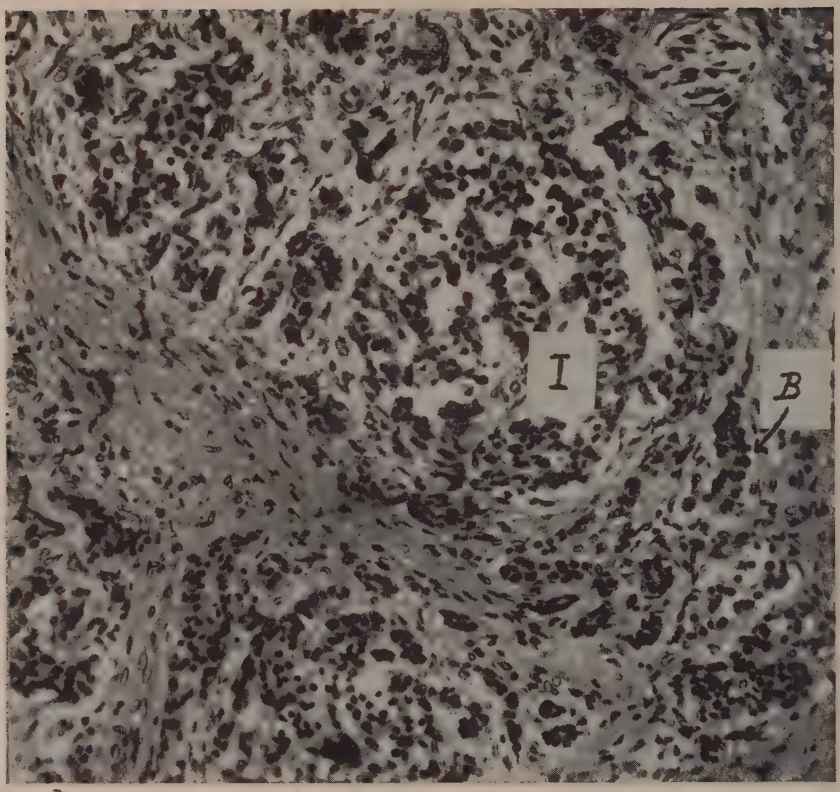

FIG. 3.

Photomicrograph × 225 from remaining portion of pancreas obtained at necropsy in patient H, 5½ months after excision of the pancreas and entire duodenum. Note fibrosis. I. Island of Langerhan's. B. Proliferating ducts. In this field there is little or no recognizable acinar tissue.

Pathology) in patients Fl. and H., 5½ and 5 months respectively after operation revealed abdominal carcinomatosis. The livers, riddled with metastases, showed no gross evidence of fatty changes. There was no gross evidence of re-establishment of pancreatic duct communications between the stumps of transected pancreas and adjacent loop of jejunum. Histologic study revealed fatty infiltration in the liver cells of the central portions of the lobules. This type of fatty infiltration is seen regularly in patients dying after a long period of debility characterized by marked reduction in food intake, as obtains in carcinomatosis. Sections of the remaining pancreas in each case showed marked fibrosis and atrophy of acinar tissue as well as invasion by carcinoma. The dense scar tissue capping the transected neck of the pancreas showed nests of carcinoma cells as well as a few small pancreatic ductules. No ulcerations in the gastrointestinal tract were present.

Discussion. The outstanding difference in reactions among the 3 patients subjected to resection of terminal portions of stomach, entire duodenum and head of pancreas, was in the character of the stool and fat absorption. Two patients exhibited fatty stools and very low absorption of ingested fat. The third patient who had typical steatorrhea before operation, had normal stools and normal fat absorption after operation when only increased flow of bile was returned to the alimentary canal (biliary flow was not completely obstructed prior to operation). The presence of an accessory pancreas in this patient could not

account for the normal stools since if this existed there could hardly have been steatorrhea for 3 weeks prior to operation, as was the case. It might be stated that no final evidence of occlusion of pancreatic juice obtains in this patient since he is still alive. However, the operation performed in this case was identical with that performed in the other two patients, namely, removal of the lower 4 cm of the stomach, entire duodenum, upper 3 cm of jejunum with transection of the neck of the pancreas at the level of the superior mesenteric vessels. The upper loop of jejunum was anastomosed to the stomach and farther along with the common duct. It thus appears that re-establishment of pancreatic ducts with the bowel could not be rapidly accomplished, if at all, bearing in mind the anatomical relationships in man. Furthermore, as previously stated, the return of normal appearing stools occurred very promptly after operation, within two weeks. It is hardly probable that under these conditions re-establishment of ducts between transected and ligated neck of pancreas with jejunum, several centimeters away could have occurred so promptly.

The above studies fail to afford a clue to any profound disturbance in the general physiologic economy of man resulting from occlusion of external pancreatic secretion, other than failure of appreciable fat absorption and incomplete digestion of raw meat in some instances. Theoretically inability to absorb fat would in time impair general nutrition if fat is an indispensable food by virtue of essential fatty acids. Furthermore, the absorption of fat soluble vitamins might be prevented and thus eventually lead to some type of avitaminosis. On the other hand, the administration of pancreatin, when necessary, could quite conceivably permit the absorption of sufficient fat, and this provided for, no other significant disturbances might be anticipated.

Conclusion. No general physiologic disturbances are observed in a patient who has survived occlusion of the external pancreatic secretion for 11 months. Previous weight loss was regained. The blood, stools, and fat absorption are normal and heavy physical labor is possible. In 2 other patients with similar occlusion, bulky fatty stools immediately developed and fat absorption was radically reduced. No other general physiologic disturbance was detected during their period of survival (5½ and 5 months respectively) except for some impairment in digestion of raw meat, death resulting from carcinomatosis. The basis for the marked variation in fat absorption following occlusion of the pancreatic juice in man remains obscure.

14179 P

Effect of Calcium Pantothenate and Para-Aminobenzoic Acid on the Gray Hair of Humans.

HAROLD BRANDALEONE, ELIZABETH MAIN, AND J. MURRAY STEELE.

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It has been reported that a deficiency of the filtrate factors of the vitamin B complex may be accompanied by graying of fur in rats and dogs. The administration of the filtrate factors and, in addition, other vitamin B factors appeared also to darken the fur in some animals.[1-5] Morgan[1,3] was the first to observe that the active substance is in the filtrate fraction of vitamin B_2, *i.e.*, it is found in the water extract of yeast, lime, or rice bran

[1] Morgan, A. F., Cook, B. B., and Davison, H. G., *J. Nutrition*, 1938, **15**, 27.

[2] Oleson, J. J., Elvehjem, C. A., and Hart, E. B., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 283.

[3] Morgan, A. F., and Simms, H. D., *J. Nutrition*, 1940, **19**, 233.

[4] Lunde, G., and Kruegstad, H., *J. Nutrition*, 1940, **19**, 321.

[5] Morgan, A. F., *Science*, 1941, **93**, 261.

after thiamine, pyridoxine, and riboflavin have been removed. This investigator also found that supplying the filtrate fraction of B_2 cured grayness of fur induced in animals by withholding the filtrate fraction. Ralli[6] found that animals with a low salt intake developed graying of fur much sooner than those on high salt intake.

There have been reports which indicate that pantothenic acid protects the fur of animals from turning gray and that its administration is followed by a return of normal color to hair that has turned gray.[3,5] Quite recently, some studies have suggested that administration of para-aminobenzoic acid and pantothenic acid to men and women with gray hair is frequently followed by return of the color of hair toward normal.[7] The wide publicity which has followed these reports made it seem desirable to plan studies of rather long duration in order to quantitate more carefully the degree of change to be expected after use of pantothenic acid and para-aminobenzoic acid.

Studies. Twenty-one elderly men and women with white or graying hair were selected from the wards of Goldwater Memorial Hospital, Third Medical Division, where they were confined with chronic diseases such as rheumatoid arthritis, general arteriosclerosis, with and without hemiplegia, and Parkinsonism. Of the 21 who began the experiment, 19 completed it; one woman refused the medication and one man left the hospital. Significant changes in the state of the disease for which the patients were admitted to the hospital were not noticed during the course of study.

Of the group of 19 patients, 7 received 100 mg calcium pantothenate, 200 mg para-aminobenzoic acid and approximately 50 g brewer's yeast daily; 5 received yeast and para-aminobenzoic acid, and 7, yeast and calcium pantothenate.* The medication was continued for 8 consecutive months; there were no apparent ill effects from any of the drugs save mild, occasional nausea probably due to the yeast.

Results. Three methods for judging change in color of hair were used: first, photographs were taken before, during, and at the end of medication; second, samples of hair were clipped from a given area at the time photos were taken; third, all patients were seen by the same 2 observers at least twice a month and notes taken of any change observed.

At the end of the study, photographs, hair samples and notes for each patient were assembled. It was found that the photographs were useless; slight changes in distance or lighting made large differences in the apparent color of hair. Hair samples were valuable in discrediting many subjectively favorable notes: a very definite color change must occur before it is apparent in the clippings. Subjective opinions are more accurate than photos and less discouraging than hair samples.

Several changes were noted. The most common was the appearance of a yellow or greenish cast to the gray hair, most often observed during the first few months, which did not always persist. Secondly, growth of scattered wiry black hairs became apparent. In several patients there was thought to be greater luster without actual change in color. In one man whose hair was sparse, there was an increase in the number of hairs. In only 2 patients was there unequivocal change in color. Both of these patients suffered from rheumatoid arthritis. Both were men, both had brown hair—the one a red-brown, the other a yellow-brown. The change in color which was observed tended toward a return to the original color. Both men noticed the change themselves. It became apparent after the drugs had been administered for a period of 2 or 3 months, thereafter increasing slowly in intensity until the drugs were stopped.

A similar study in younger individuals is under way.

Conclusions. In a group of 19 elderly individuals with gray hair, a significant color change was noted during a period of intensive vitamin therapy in only 2 individuals; these subjects received both calcium pantothenate

[6] Ralli, E. P., Clarke, D. H., and Kennedy, E., *J. Biol. Chem.*, 1941, **141**, 105.

[7] Sieve, B. F., *Science*, 1941, **94**, 257.

* We are indebted to Merck & Co., Inc., through the offices of Dr. D. F. Robertson for furnishing us with the rather large quantities of calcium pantothenate and para-aminobenzoic acid necessary for this study and to the Vitamin Food Co., Inc., for the brewer's yeast.

and para-aminobenzoic acid, together with brewer's yeast. Both were men. The fact that the only 2 substantiated results occurred in the group receiving both calcium pantothenate and para-aminobenzoic acid may be of importance. Some questionable changes were noted when one or the other drug was given alone.

14180

Transmission of West Nile Virus by Infected *Aedes albopictus*.

C. B. PHILIP AND J. E. SMADEL.

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The West Nile virus,[1] a neurotropic agent related immunologically to the viruses of St. Louis and Japanese encephalitis,[2] has apparently caused human infection in widely scattered communities in equatorial Africa.[3] Comparatively little is known about the natural mode of transmission of this agent;[1-3] however, the importance of arthropod vectors in the spread of other neurotropic viruses (see references cited in [4]) warranted an investigation of their role in West Nile encephalitis. Young hamsters have been shown recently to be suitable for use in experiments dealing with the insect transmission of West Nile virus.[4] The results presented at this time indicate that *Aedes albopictus* can become infected by feeding on hamsters with West Nile encephalitis and that following an incubation period such mosquitoes can transmit the virus to hamsters by biting. Further tests of transmission with this and other species of mosquitoes and with certain argasid ticks and triatomid bugs are in progress.

Three hamsters, weighing 30 to 40 g, were injected intraperitoneally with a suspension of mouse brain containing 10^8 mouse cerebral lethal doses (CLD) of virus. Twenty-four hours later the animals were anesthetized with sodium barbital given intraperitoneally in doses of 50 mg per 100 g body weight. During the succeeding 2 hours 76 *Aedes albopictus* were fed on the shaven abdomens of the hamsters. Pooled plasma obtained from the hamsters immediately after the meal contained $10^{4.6}$ CLD of virus; the donors died on the 6th, 7th, and 8th days respectively after intraperitoneal inoculation. Ten insects were killed with tobacco smoke at the end of the donor meal and triturated in 2 cc of saline solution containing 2% normal guinea pig serum; this suspension was centrifuged at 3,000 r.p.m. for 10 minutes after which the clear, claret-colored fluid of the midzone was removed with a capillary pipette. All 6 of the mice injected with this suspension succumbed in 5 to 8 days; suspensions of brain tissue from 2 of these were bacteriologically sterile and were infectious for mice when injected intracerebrally.

The average weight of an adult *A. albopictus*, determined by weighing groups of 40 insects of the same stock and of about the same age as those used in the present experiment, was 2.31 mg when engorged and 1.31 mg when without blood. Therefore, suspensions of the type just described containing mosquito tissue and donor blood were considered as being 10^{-2} dilutions of either insect material or blood depending on which substance was being tested. Although the titration of virus in this lot of mosquitoes was not carried higher than 10^{-2} it was assumed that its titer was the same as that of the donor plasma, namely $10^{-4.6}$.

Forty-three mosquitoes of this lot, after storage for 10 days at 90°F, bit 2 baby hamsters which weighed 25 and 30 g, respec-

1 Smithburn, K. C., Hughes, T. P., Burke, A. W., and Paul, J. H., *Am. J. Trop. Med.*, 1940, **20**, 471.

2 Smithburn, K. C., *J. Immunol.*, 1942, **44**, 25.

3 Smithburn, K. C., and Jacobs, H. R., *J. Immunol.*, 1942, **44**, 9.

4 Watson, D. W., and Smadel, J. E., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 101.

TABLE I.
Transmission of West Nile Virus by Infected *Aedes albopictus*, Lot III.

Time after donor meal, days	Virus in mosquitoes*	Hamsters bitten	No. of insects fed	Disease in hamsters	Virus isolated from hamsters†
Immediately	>10-2 (approx. 10-4.5)	None			
10	10-5.6	a§	43	+	+
		b		+	+
18	10-4.6	a	5	+	+
		b	5	?	—
		c	16	+	+
21		a	3	—	‡
24		a	7	+	+
26		a	3	+	+
40		a	2	±	—

* The amount of virus in mosquitoes was determined by intracerebral titration in mice of suspensions of ground insects.
† Hamster brain tissue was injected intracerebrally in mice for isolation of virus.
‡ Not attempted.
§ Each letter designates different hamster.

tively. Ten of the engorged mosquitoes were ground in 2 cc of diluent and the suspension was titrated by injecting groups of 3 mice intracerebrally with serial 10-fold dilutions; the titer was $10^{-5.6}$ One of the bitten hamsters developed weakness and tremors on the 6th day and was moribund on the 7th while the other sickened on the 9th day and was sacrificed on the 10th. An infectious agent pathogenic for mice was isolated from the bacteriologically sterile brain tissue of each hamster. Furthermore, histo-pathological studies of sections of brain from each animal showed lesions of the type seen in hamsters with West Nile encephalitis.[4] Finally, identification of the first mosquito-transmitted strain of virus was made by preparing an antigen from mouse brains of the 4th passage and demonstrating its capacity to fix complement with specific antibodies in the serum of a hamster hyperimmune to West Nile virus.[5]

A similar successful feeding experiment was made with this same lot of infected mosquitoes after 18 days at 90°F, at which time the titer of virus in the insects was $10^{-4.6}$ In this and later experiments transmission of infection by biting was considered adequately proven if the bitten hamster developed signs of central nervous system disease within 6 to 10 days and if its bacteriologically sterile brain tissue when injected intracerebrally into mice was capable of inducing a clinical disease like West Nile encephalitis. Insects were stored at 75°F after the 18th day because of the relatively high mortality among the mosquitoes maintained at a constant temperature of 90°F. A summary of the results obtained in feeding experiments carried out with this lot of mosquitoes 10 to 40 days after the initial blood meal is presented in Table I. It is seen that 6 of the 9 hamsters bitten by this group of insects were successfully infected. Furthermore, disease was induced by the feeding of as few as 3 or 5 of these mosquitoes, although positive results were not regularly obtained following the biting of such few insects.

Transmission of West Nile virus was not accomplished in 2 additional experiments similar to the one just described. In one instance, the engorged mosquitoes contained insufficient virus to be detected on intracerebral inoculation in mice. In the other, although a small amount of virus was initially present (one of 6 mice became infected), no virus was demonstrable in mosquitoes stored at 75°F for 10 and 18 days.

Summary. The virus of West Nile encephalitis can be transmitted in the laboratory by the bites of infected *Aedes albopictus*.

[5] Havens, W. P., Watson, D. W., Green, R. H., Lavin, G. I., and Smadel, J. E., *J. Exp. Med.*, 1943, **77**, 139.

14181

Action of *l*-Ascorbic Acid upon the Isolated Frog Heart.*

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It was recently reported that hydrogen peroxide is the cause of the positive inotropic and of the irreversible systolic effect upon the frog heart of ascorbic acid solutions.[1] The evidence available in the literature indicates that the hydrogen peroxide formation in ascorbic acid solutions is related to the dehydrogenation of the molecule, and, more specifically, to the catalytic action of copper in this process.[2] It is known that the catalytic action of copper can be prevented by the formation of complexes between the metal and suitable substances like protein[3] or diethyldithiocarbamate.[4] Serum globulin and sodium diethyldithiocarbamate were therefore used to determine whether copper inhibitors were able to diminish or prevent the action of *l*-ascorbic acid solutions upon the frog heart.

Method. 50 to 100 mg of *l*-ascorbic acid were dissolved in one cc of distilled water; and this solution, after neutralization with sodium bicarbonate, was diluted to an initial concentration of approximately 1:10,000 with bicarbonate buffer solution (NaCl 85.4; KCl 1.9; $CaCl_2$ 0.99; $NaHCO_3$ 27.0 mM/L) saturated with 95% oxygen and 5% carbon dioxide. The pH of the solution was between 7.5 and 7.8. The room temperature varied between 20 and 25°C. In the experiments with inhibitor, this was dissolved in the bicarbonate buffer solution previous to the addition of the *l*-ascorbic acid. The serum globulin was prepared in the laboratory of Dr. E. J. Cohn and consisted of 90% gamma-globulin and 10% beta-globulin.

The frog hearts were isolated as previously described,[1] and the bicarbonate buffer solution was continuously perfused through the cannula

TABLE I.
(Isolated frog hearts.)
Inhibition by Serum Globulin and by Sodium Diethyldithiocarbamate of *l*-Ascorbic Acid Destruction and Corresponding Modification of Heart Action of *l*-Ascorbic Acid Solutions.

Inhibitor		*l*-Ascorbic acid									
		Concentration in mg %				% destruction			Systolic effect		
			After			After			After		
Name	Conc. in mg %	Initial	1 hrs	2 hrs	3 hrs	1 hrs	2 hrs	3 hrs	2 hrs	3 hrs	
Serum globulin (consisting of 90% γ-globulin and 10% β globulin)	1.0	10.0	7.2	4.3		28	57		complete		
	2.0	14.4	8.6	4.7		40	67		”		
	5.0	10.8	8.0	4.8	2.8	26	56	74	begins	complete	
	10.0	10.5	9.0	8.7	8.0	14	17	24	none	doubtful	
	20.0	8.3	8.3	8.3	8.3	0	0	0	”	none	
Sodium diethyl dithiocarbamate	0.1	10.0	9.8	9.0	8.7	2	10	13	”	”	
	0.1	10.0	9.6	9.2	8.6	4	8	14	”	”	
	1.0	10.9	10.3	10.1	9.6	5	7	12	”	”	
	1.0	10.9	10.3	10.2	9.7	5	6	11	”	”	

* This work was carried out under the auspices of the University Committee on Pharmacotherapy.

1 Krayer, O., Linstead, R. P., and Todd, D., *J. Pharm. and Exp. Therap.*, 1943, **77**, 113.

2 Lyman, C. M., Schultze, M. O., and King, C. G., *J. Biol. Chem.*, 1937, **118**, 757; Dekker, A. O., and Dickinson, R. C., *J. Am. Chem. Soc.*, 1940, **62**, 2165; Steinman, H. G., and Dawson, C. R., *J. Am. Chem. Soc.*, 1942, **64**, 1212.

3 Ettisch, G., Sachsse, H., and Beck, W., *Biochem. Z.*, 1931, **230**, 68.

4 McFarlane, W. D., *Biochem. J.*, 1936, **30**, 1472; Stotz, E., Harrer, C. J., and King, C. G., *J. Biol. Chem.*, 1937, **119**, 511.

at a rate of 2 to 2.5 cc per minute, with a gas mixture of 95% oxygen and 5% carbon dioxide bubbling through the fluid in the cannula.

The ascorbic acid determinations were made with the 2,6-dichlorindophenol method. The approximate concentration of hydrogen peroxide was determined by its luminescent reaction with 3-aminophthalhydrazide.[5,1]

Results. Without inhibitor, solutions of *l*-ascorbic acid with an initial concentration of 1:10,000 (in Ringer solution not specifically freed from copper[6]) caused systolic standstill within one to 2 hours, 80 to 90% of the *l*-ascorbic acid being destroyed within this period. Hydrogen peroxide accumulated in the solutions to reach concentrations of the order of 1:100,000 to 1:300,000.[1] Serum globulin or sodium diethyldithiocarbamate in appropriate concentrations inhibited the destruction of *l*-ascorbic acid and the solutions had little or no action upon the heart (Table I). The concentration of hydrogen peroxide was very low in the solutions in which the ascorbic acid destruction was markedly inhibited. The characteristic luminescence with 3-aminophthalhydrazide was either very weak or entirely absent, indicating concentrations of hydrogen peroxide of only one in 5 to 10 million.

These experiments show that *l*-ascorbic acid solutions (in the presence of oxygen and at pH 7.5 to 7.8) do not exhibit their characteristic positive inotropic and systolic effect upon the frog heart in the presence of copper inhibitors. Under this condition the hydrogen peroxide formation in the solution is insufficient to cause the heart effect.

The author is indebted to Dr. Sydney Ellis for the *l*-ascorbic acid estimations.

5 Schales, O., *Ber. d. deutsch. Chem. Ges.*, 1938, **71**, 447.

6 Peugnet, H. B., *Science*, 1939, **90**, 162.

14182

Preparation of and Cardiovascular Response to Neo-synephrine in Oil.

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Neo-synephrine HCl (laevo a-hydroxy-b-methyl-amino-3-hydroxy ethyl benzene hydrochloride) when used parenterally has been found to be a useful adjuvant in spinal anesthesia[1,2,3,4] and in cyclopropane anesthesia.[5] This substance raises and maintains blood pressure over a considerable period of time. Side reactions such as nervousness, anxiety, etc., are comparatively rare. In addition, neo-synephrine HCl has been used successfully in the treatment of some forms of shock.[6,7]

Blood pressure within a normal range can be maintained under various conditions by repeated intravenous injections of small amounts of neo-synephrine HCl, or by subcutaneous or intramuscular injections repeated as necessary. The latter methods of administration tend to establish a depot from which absorption takes place rather slowly into the blood stream. Inasmuch as substances in oil, when injected parenterally, are absorbed more slowly than those in aqueous solutions, the

1 Lorham, P. H., and Oliverio, R. M., *Anesth. and Analg.*, 1938, **17**, 44.

2 Brunner, R. S., and deTakats, G., *Surg., Gyn., and Obst.*, 1939, **68**, 1021.

3 Bitterich, N. M., *Anesth. and Analg.*, 1939, **18**, 29.

4 Cranston, Elizabeth M., and Bieter, R. N., *J. Pharm. Exp. Therap.*, 1940, **68**, 141.

5 Orth, O. S., Leigh, M. D., Mellish, C. H., and Stutzman, J. W., *J. Pharm. Exp. Therap.*, 1939, **67**, 1.

6 Mahaffey, H., *Anesth. and Analg.*, 1939, **18**, 196.

7 Johnson, Carl A., *Surg., Gyn., Obst.*, 1937, **65**, 458.

TABLE I.
Toxicity of Neo-Synephrine HCl and Neo-Synephrine in Oil Compared.

Drug	Dose mg/kg*					
	30	35	40	45	50	55
Neo-Synephrine HCl	5/30†	10/30	14/30	21/30	27/30	—
Neo-Synephrine in Oil	—	8/30†	9/30	14/30	20/30	25/30

* All injections were made subcutaneously into albino rats. Survival was recorded for 4 days. The LD 50 was obtained by plotting the Log Dose against % mortality.
† No. dead/ No. injected.

intramuscular injection of neo-synephrine in oil should result in a more prolonged vasoconstriction. Accordingly, we have prepared and are reporting the results obtained with such a solution.

Methods. Neo-synephrine base, insoluble in oil, was found by Heyn and Geiter[8] to be soluble in peanut oil containing oleic acid. The solution employed in this investigation contained 0.82% neo-synephrine base dissolved in peanut oil with 10% oleic acid as the solubilizing agent. The amount of base present is equivalent to that contained in a 1% neo-synephrine HCl aqueous solution.

The effects on blood pressure and heart rate were determined on the unanesthetized dog following subcutaneous and intramuscular injections. Blood pressure was measured by means of a mercury manometer to which was attached a cuff specially designed to fit snugly the upper foreleg or thigh. In all instances, the response to the injection of neo-synephrine in oil was compared with that resulting from the injection of an equivalent amount of an aqueous neo-synephrine HCl. The toxicity was determined by subcutaneous administration to albino rats. All experimental animals were housed in air-conditioned quarters with a constant temperature of 75°F and a relative humidity of 35%. Thirty animals with an average weight of 200 g and of equal sex were injected at each dosage level. Neo-synephrine HCl as a 1% aqueous solution was injected in the following amounts: 30, 35, 40, 45, and 50 mg/kg. Neo-synephrine in a 1% equivalent oil solution was injected in the following amounts: 40, 45, 50, and 55 mg/kg.

Results. In the trained dog, atropinized and without an anesthetic, a definite rise in blood pressure was produced with as little as 0.05 mg/kg of neo-synephrine HCl or an equivalent amount of neo-synephrine in oil. Intramuscular injections of 0.1-0.25 mg/kg of neo-synephrine in oil was followed by a rise more sustained than that caused by an equal amount of aqueous neo-synephrine HCl. Whereas the latter solution in the above amounts, caused rises in pressure lasting as long as 90 minutes, those in oil caused rises lasting as long as 197 minutes. Injections of 0.5 mg/kg of either solution were not well tolerated and frequently caused such reactions as:

1. Pilomotor action over shoulders and tail.
2. Distinct hyperpnea.
3. Occasional emesia near or during the peak of the blood pressure rise.

Generally these reactions were associated with blood pressure elevations of approximately 75 to 130 mm Hg above the normal resting systolic pressure.

In the unatropinized dog, there is a marked bradycardia simultaneous with the rise in blood pressure, particularly in the diastolic pressure. With near threshold doses, cardiac slowing may be the only demonstrable response. The administration of neo-synephrine in oil leads to a more prolonged response than does an equivalent amount of neo-synephrine HCl by either subcutaneous or intramuscular routes. Thus, neo-synephrine HCl injected subcutaneously in doses of 0.1 mg/kg produced rises in systolic pressure up to 70 mm Hg with elevations lasting as long as 225 minutes. Under the same conditions neo-synephrine in oil caused rises up to 64 mm Hg with the duration of some elevations lasting as long as 340 minutes. Intramuscular injections gave results similar to those cited above with the exception that the duration of the elevated pressure was somewhat more

[8] Heyn, M., and Geiter, C. W., unpublished data.

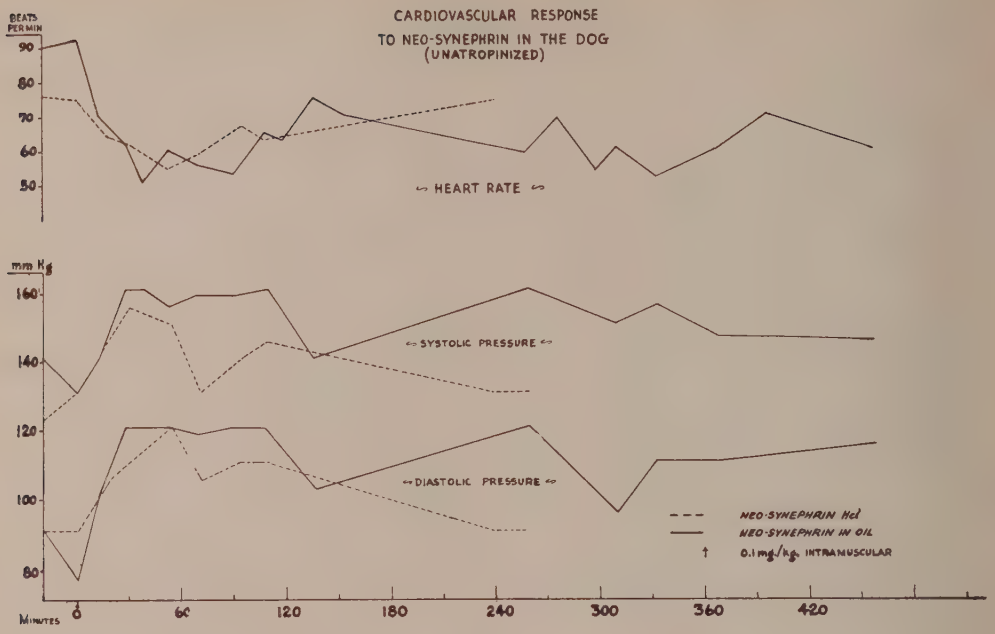

FIG. 1.

prolonged in most instances. The cardiac slowing was more prolonged than the rise in blood pressure following the injection of neo-synephrine in oil by either parenteral route. Fig. 1 gives in some detail the comparative responses obtained in one animal. This is representative of the series.

When administered subcutaneously in a series of albino rats the LD 50 of the neo-synephrine in oil is 45.9 mg/kg. The LD 50 of the neo-synephrine HCl under similar conditions is 40.7 mg/kg (equivalent in terms of neo-synephrine base to 33.4 mg/kg) (Table I). Therefore toxicity of the neo-synephrine in oil would be 28% less than that of the aqueous hydrochloride.

Summary. 1. Neo-synephrine base is soluble in peanut oil containing oleic acid. 2. Neo-synephrine in oil injected subcutaneously or intramuscularly brings about a more sustained rise in blood pressure than does a similar injection of an equivalent amount of aqueous neo-synephrine HCl. 3. The threshold dose for both forms of neo-synephrine is about 0.05 mg/kg in the dog. Maximal responses are usually obtained with doses of 0.5 mg/kg by either subcutaneous or intramuscular injection. The latter dosage level is not well tolerated and is frequently followed by undesirable side reactions. 4. Neo-synephrine in oil is 28% less toxic than the aqueous neo-synephrine HCl equivalent. The slower rate of absorption from oil is the probable cause for the lower toxicity.

14183

The Liberation of Biotin from the Avidin-Biotin Complex (AB).

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In investigations of the relationship of avidin and biotin the means by which biotin bound to avidin may become physiologically active has remained obscure. In the digestive tract the avidin-biotin bond is firm since ingested egg white prevents absorption of biotin. This tight link is surprising in view of the protein-like nature of avidin and the presence of proteolytic enzymes in the stomach and intestine. Parenterally administered, however, avidin-biotin (AB) is apparently broken down since egg white injury may be cured by injection of avidin containing some bound biotin.[1]

AB seems to be more resistant to chemical action than avidin itself. György, Rose, and Tomarelli have shown[2] that while the ability of avidin to combine with biotin is greatly reduced under conditions of temperature and acidity found in the stomach, AB not only is unaffected, but seems in some measure to protect the avidin. Except for steaming, which readily and completely releases biotin, only a few cases of its liberation from avidin have been noted. In experiments where avidin containing AB was irradiated in the presence of riboflavin a considerable portion of the biotin was released.[2] A different type of reaction is observed when biotin sulfone in excess is added to AB.[3] It may be shown by yeast growth tests that biotin is present in free form apparently replaced in the AB complex by biotin sulfone. To enlarge our information on the behavior of AB we have subjected it to various treatments simulating the physiological reactions—enzymatic or oxido-reductive—which might be responsible for liberating it in the body.

Experimental. The methods of biotin and avidin assay are those of Eakin, Snell, and Williams[4] except that *l*-asparagine was substituted for *l*-aspartic acid in the biotin-free medium since the latter acid was not procurable. The avidin used has been either egg white or a purified concentrate. The biotin was crystalline biotin methyl ester. In most of the experiments AB has been prepared with the avidin in slight excess so that small amounts of liberated biotin might be more accurately estimated.

A study of the effect of proteolytic enzymes on AB was made, since, though we had presumed that it was unaffected by the proteolases of the digestive tract, no direct observation of the fact had been made. Solutions of egg white and avidin containing approximately 0.2 μg of biotin per cc were digested at 38°C with 0.2% pepsin at a pH of 1.8 for 24 and 48 hours. After 48 hours there was a trace of free biotin but it was less than 1% of the total amount present. A 24-hour egg white pepsin digest was adjusted to pH 8 and digested for an additional 24 hours with 0.2% trypsin without further liberation of biotin. Similarly a 24-hour avidin pepsin digest was brought to pH 8 and incubated with 0.4% pancreatin. No free biotin was produced. These mixtures were dialyzed to test whether the avidin had been hydrolyzed and the biotin be bound still by some dialyzable fragment of it. The results were negative. Because of the effectiveness of papain as a proteolytic agent an attempt was made to liberate biotin by its means. An egg white solution containing 0.1 μg of biotin with 0.2 mg papain and 0.01 cc of 1.2% HCN per cc as activator was incubated at 70°C for 24, 48, and 96 hours. It was then dialyzed for 24 hours. There was

1 György, P., and Rose, C. S., *Science*, 1941, **94**, 261.

2 György, P., Rose, C. S., and Tomarelli, R., *J. Biol. Chem.*, 1942, **144**, 169.

3 Dittmer, K., du Vigneaud, V., György, P., and Rose, C. S., to be published.

4 Eakin, R. E., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, 1941, **140**, 535.

no dialyzable biotin and no yeast active biotin in the residue although, after 96 hours, 80% of the egg white protein nitrogen had been converted to non-protein nitrogen. One might have expected that temperature alone would have liberated the biotin in this case.

Release of biotin from avidin by means of tissue enzymes seemed to be a probable explanation of AB activity within the body. Beef liver, kidney, and muscle were used, the same procedure being followed in the 3 cases. Ground tissue (1 g), 1 cc of avidin-biotin containing 1 μg of biotin and 2 cc of an isotonic solution of sodium phosphates (pH 7.4) were mixed, overlaid with benzene for protection against bacterial action and incubated at 38°C for 48 hours. After incubation the solutions were diluted to 10 cc with saline, centrifuged and filtered. The filtrates contained no free biotin.

In blood AB was equally recalcitrant. Both oxygenated and reduced blood were studied since the degree of oxidation might be a factor in the reaction. Mixtures of 2.5 cc of citrated human blood with 1 cc of AB containing 0.6 μg of biotin and 1.5 cc of normal saline were put in tonometers one of which was filled with oxygen, the other with nitrogen, and incubated at 38°C for 24 hours. The mixtures were then dialyzed in the ice box for 24 hours, the reduced sample being protected with oil. No biotin was found in the dialysate nor any free biotin in the residue. A further experiment was made using methylene blue and cysteine to modify the degree of oxidation of the blood. The following systems were studied:

Blood +	AB +	5% cysteine +	5% M.B. +	saline
cc	cc	cc	cc	cc
2.5	1	0	0	1.5
2.5	1	0.5	0	1
2.5	1	0	1	0.5
2.5	1	0.5	1	

In this case defibrinated rabbit's blood was used. Incubation and dialysis were carried out as in the preceding experiment and the results were similarly negative.

Extensive oxidation of biotin produces compounds inactive or only slightly active in promoting yeast growth, as shown by Brown and du Vigneaud.[5] For our purposes a method used by Nielsen, Shull, and Peterson[6] offered possibilities. These authors found that the mild oxidation of biotin with 0.3% hydrogen peroxide at pH 3 used by Shull, Hutchings, and Peterson[7] produced a biotin derivative still active in promoting yeast growth though inactive for *Lactobacillus casei* and rats. We have used this method for biotin and AB with biotin concentrations of 0.05 to 0.1 μg per cc. Control samples were run, omitting only the H_2O_2 from the procedure. Total biotin of AB was determined by liberation of the biotin by steaming. This steaming was done before the MnO_2 added to decompose the excess H_2O_2 was removed. Otherwise avidin with the biotin bound to it was absorbed on the MnO_2 and lost on filtration. We found a greater loss of biotin activity than did the authors of the method, but our purpose was accomplished. A definitely measurable quantity of a substance with yeast-growth promoting ability was liberated from AB. The experiments were repeated using larger amounts of H_2O_2. A concentration of 0.45% liberated a greater amount of "biotin." A higher concentration (0.6%) offered no further advantage. With the 0.45% level of H_2O_2 amounts of free material up to 20% of the biotin originally present were found.

A sample of peroxide treated AB was dialyzed against 0.9% NaCl. Almost all of the liberated biotin was found in the dialysate. At pH 7 only a small amount of biotin was released from AB by oxidation (2 to 3% of that originally present) nor was there much reduction in the total biotin content. At this pH free avidin is not readily destroyed as it is at pH 3. When additional avidin was added to the oxidized free biotin or to the liberated biotin they were completely rebound. The amount of avidin required was the same as was required for an amount of the original biotin producing the same amount of yeast growth. The decrease in activity of the solutions, therefore, does not seem to be due to conversion of all

[5] Brown, G. B., and du Vigneaud, V., *J. Biol. Chem.*, 1941, **141**, 85.

[6] Nielsen, E., Shull, G. M., and Peterson, W. H., *J. Nutrition*, 1942, **24**, 523.

[7] Shull, G. M., Hutchings, B. L., and Peterson, W. H., *J. Biol. Chem.*, 1942, **142**, 913.

TABLE I.

Material tested	Biotin activity (μg/cc)				
	Original sol.	Sol. treated without H_2O_2	Oxidized solution		
			0.3% H_2O_2	0.45% H_2O_2	0.6% H_2O_2
Biotin	0.089	0.060	0.030	0.022	0.030
AB	0 (0.091)*	0	0.0040	0.019	0.011
AB	0 (0.044)	0 (0.040)		0.0024 (0.037)	

* The figures in parenthesis are total biotin (free + bound).

of the biotin to a less active form but to complete loss of activity of a portion of it.

Discussion. The experiments here described indicate that enzymatic processes are not responsible for liberation of biotin from avidin in the body. The metabolism of AB is more likely linked with oxidation-reduction systems. The oxidation method used in these experiments does not presume to duplicate a physiological process, but the results indicate that oxidation is a means of attack on an otherwise refractory compound. Recent work has shown that biotin is only one of a number of related compounds concerned in metabolic processes. Oppel[8] has described avidin-combining and avidin-non-combining fractions of urine of which the former seemed more closely related to biotin intake. That fraction might be a modified biotin such as that obtained in these experiments which does not differ appreciably from true biotin in its ability to combine with avidin or its yeast growth promoting activity. Burk and Winzler[9] have suggested that biotin and its modifications may act in alternation between avidin-combining and avidin-non-combining form. Further investigations of the various biotin related compounds with respect to their avidin-combining power, their growth promoting effect on various organisms and the methods of conversion from one to the other will be helpful in understanding the function of biotin.

Summary. 1. Under the experimental conditions used biotin could not be liberated from combination with avidin by the proteolytic enzymes—pepsin, trypsin, pancreatin, and papain—nor by incubation with liver, kidney, muscle, or blood. 2. Biotin could be liberated from the avidin-biotin complex by oxidation with 0.45% H_2O_2. Amounts of yeast active material equivalent to 10 to 20% of the biotin originally bound were found.

[8] Oppel, T. W., *Am. J. M. Sc.*, 1942, **204**, 856.

[9] Burk, D., and Winzler, R. J., *Science*, 1943, **97**, 57.

14184 P

Use of Sulfonamides in Treatment of Experimental *Clostridium welchii* Infection.

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The efficacy of oral sulfonamide therapy in experimental *Cl. welchii* infection in mice has been discussed previously.[1] In that study, it seemed desirable to produce a concentration of drug in the blood of 10-20 mg %. This required that the drug be administered in the food 3 to 4 days before inoculation with *Cl. welchii*, and consequently the results obtained were viewed more from the standpoint of prophylaxis than treatment, although all the animals showed a generalized infection 6 to 18 hours after inoculation, and had large lesions at the site of inoculation.

The present study was undertaken in order to evaluate more accurately the treatment of this infection, and to determine the effect of high local concentrations of the drugs on the lesion which resulted at the site of inoculation.

Infection was produced as previously described[1] by intramuscular inoculation into the inner aspect of the thigh of a whole, unwashed, 18-hour culture. No other necrotizing agent was used. The amount of inoculum used was sufficient to kill 90-100% of the untreated animals. This amount had been determined by previous standardization (usually 0.25 ml of a 1:4 dilution of culture was used), and as a further check 10 control animals were inoculated with each group of animals studied.

Various suspensions of sulfadiazine, sulfathiazole, and sulfanilamide were used; 0.25 ml or 0.1 ml of a 20%, 10%, 5%, or 1% suspension was injected subcutaneously into the inguinal region of the infected leg, before, at the same time, and at 1-, 2-, and 3-hour intervals after inoculation with *Cl. welchii*.

The suspensions of the drug were prepared in honey and water. For a 20% suspension: 2 g of drug was finely pulverized in a mortar and 2.4 ml of honey added gradually with continued grinding. When a smooth paste had been prepared 7.6 ml of water was added drop-wise with stirring. Smoother suspensions could be obtained with sulfadiazine than with the other drugs. All the suspensions tended to precipitate slowly, but if shaken frequently, they proved entirely satisfactory for injection with a 26-gauge needle.

At first it was thought that with this technic of injecting the drug near the site of inoculation it would be possible to study the local effect of the drug. Actually, it proved to be a means of producing high sustained concentrations of the drugs in the blood. Charts 1 and 2 give the curves of the concentrations of the drugs (free-sulfonamide) in the blood when 20% and 5% suspensions of the drugs were injected. Each point on the curve represents the average of 10-12 determinations. Since equipment for micro-determinations was not available, it was necessary to sacrifice an animal for each determination.

The trends of the curves are not altered appreciably by the amounts of drug conjugated as the acetyl-sulfonamide, nor by varying the concentration of honey used. The initial rise, therefore, seems to be dependent upon the water content. The tremendous concentrations of drug in the blood obtained with the 20% suspension of sulfanilamide proved so toxic that most of the animals died within 6-10 hours.

It was of interest to know which drug, what dosage, and what height of blood concentration was necessary to produce the best survival rates in infected animals. The results

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† Joseph B. DeLee Fellow 1942-43.

The sulfonamides used in this study were furnished through the courtesy of Lederle Laboratories, Inc. (sulfadiazine), and the Department of Medical Research, Winthrop Chemical Co. (sulfathiazole and sulfanilamide).

1 Hac, L. R., Eilert, M. L., and Adair, F. L., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 108.

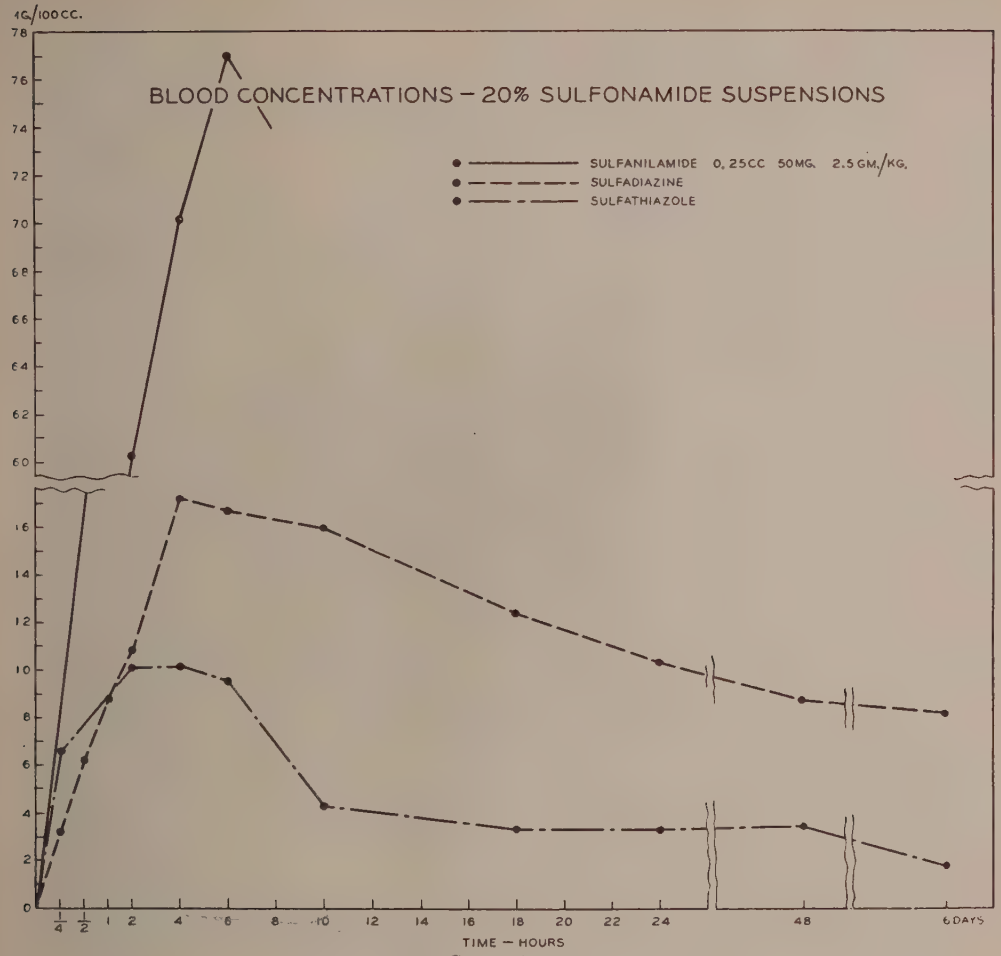

CHART 1.

(averages of repeated experiments) obtained with various doses of the drugs, when a single injection of the drug was made at the same time as, and at 1-, 2-, and 3-hour intervals after the inoculation with *Cl. welchii* are given in Table I.

Sulfadiazine seems to be only very slightly, if at all, more efficacious than sulfathiazole, but both are considerably more effective than sulfanilamide. Repeated injections of the drug in order to maintain the initial high concentration of drug in the blood proved no more efficacious than a single dose, and, surprisingly, a 1 mg dose protected as effectively as a 50 mg dose.

No better survival was obtained when the drug was injected into the inguinal region of the infected leg than when similarly injected into the non-infected (opposite) leg, which would seem to indicate that protection was due largely to the concentration of drug in the blood rather than to the local concentration.

Protection was markedly decreased by a delay of one hour, and there was very little protection after 2 hours.

Each animal had a large lesion at the site of inoculation, similar to that described when oral therapy was used. Most of the animals were killed at the end of one week, but ob-

CONCENTRATIONS OF DRUGS IN THE BLOOD. 5% SULFONAMIDE SUSPENSIONS

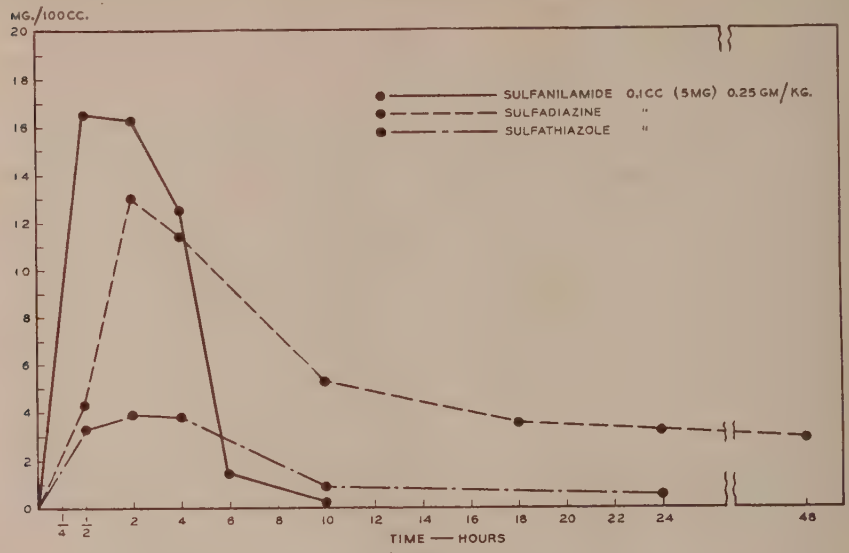

CHART 2.

servations of the lesion were made in some for as long as 6 weeks when healing had usually become complete.

Summary. 1. A suspension of sulfonamides injected subcutaneously into the inguinal region of mice produced high sustained concentrations of the drug in the blood. 2. A single dose of a suspension of sulfathiazole or sulfadiazine injected immediately following inoculation with *Cl. welchii* protected 45-53% of the mice. A 1 mg dose gave as much protection as a 50 mg dose. A delay in therapy of 1 or 2 hours markedly reduced the protective action. Sulfanilamide gave very little protection.

TABLE I.
Effect of Drug, Dosage, and Time of Therapy on *Cl. welchii* Infection.

Time	Dose, mg	No. animals	% survival		
			Sulfathiazole	Sulfadiazine	Sulfanilamide
3 hrs before *welchii*	50	30		97	
Same time as "	50	100	45	53	
" " " "	5	100	40	48	30*
" " " "	1	100	50	51	10*
1 hr after "	50	50	20	38	0*
2 " " "	50	50	14	16	
3 " " "	50	25	12	8	

* 30 animals.

14185 P

Penicillin in Treatment of Experimental *Clostridium welchii* Infection.

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Gas gangrene remains one of the most serious complications of war wounds in spite of present methods of chemotherapy, serum therapy, and surgery. The mortality is high and the rapidity with which the infection progresses makes treatment difficult. Several anaerobes or mixtures of anaerobes are known to cause the infection, but the one encountered most frequently is *Clostridium welchii*. Previously, we had made a study of the use of sulfonamides in the treatment of experimental *Cl. welchii* infection in mice.[1,2] It was possible to protect over 50% of the infected animals with sulfadiazine or sulfathiazole, but all of them had a huge lesion at the site of the inoculation from which *Cl. welchii* could be isolated 4 to 5 weeks after inoculation. Penicillin has been used in the present study in an attempt to increase the survival rate of the infected animals as well as to study its effect upon the local lesion.

It has been only recently that penicillin has been used in the treatment of infections, and information concerning its use in *Cl. welchii* infection is very meager. Gardner[3] found that in liquid medium penicillin completely inhibited the growth of *Cl. welchii* at a dilution of 1 in 6000. With highly purified penicillin, Florey and Jennings[4] found the highest dilution which completely inhibited growth was 1 in 4 million, with partial inhibition at a dilution of 1 in 8 million. Recently, McIntosh and Selbie[5] stated that penicillin injected at the site of infection within 3 hours of infection was a powerful prophylactic against *Cl. welchii* infection in mice.

Mice and guinea pigs have been used in our study. A typical intramuscular infection was produced as previously described[1] by the inoculation into the inner aspect of the thigh of a whole, unwashed, 18-hour culture. No other necrotizing agent was used. Within an hour after inoculation, the leg had become inflamed. There was considerable swelling after 2 hours and some dark discoloration a few hours later. The amount of inoculum used was sufficient to kill 90-100% of the untreated animals. This amount had been determined by previous standardization (usually 0.25 ml of a 1:3 dilution of culture was used), and as a further check 10 control animals were inoculated with each group of animals studied.

TABLE I.
Penicillin—Effect of Varying Dosage
Single Dose—Same Leg.

Units	No. animals	% survival
500	25	100
50	100	98
25	100	81
15	75	72
5	50	54

The penicillin, 0.25 ml, containing the desired number of Florey units, was injected subcutaneously into the inguinal region of the infected leg, or of the opposite leg. The effect of immediate and delayed treatment (1, 2, and 3 hours) was determined.

The results (averages of repeated experiments) obtained in mice, when a single injection of various doses of penicillin was made at the same time as the inoculation with *Cl. welchii*, are given in Table I.

* A. B. Kuppenheimer Foundation.

† Joseph B. DeLee Fellow 1942-43.

The penicillin used in this study was furnished through the courtesy of Merck & Co.

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3 Gardner, A. D., *Nature*, London, 1940, **146**, 837.

4 Florey, H. W., and Jennings, M. A., *Brit. J. Exp. Path.*, 1942, **23**, 120.

5 McIntosh, J., and Selbie, F. R., *Lancet*, 1942, Dec. 26, **243**, 750.

Small doses of penicillin repeated every 4 hours over a period of 48 hours gave as good results as single large doses. (Table II.)

Better results were obtained when the drug was injected into the inguinal region of the infected leg than when similarly injected into the non-infected (opposite) leg. (Table III.)

TABLE II.
Penicillin—Effect of Repeated Doses q. 4 Hrs.

Units	No. animals	% survival	
		1 dose	8 doses
25	50	80	96
15	75	75	92
5	50	54	92

TABLE III.
Penicillin—Comparison of Same and Opposite Leg.

Units	No. animals	% survival	
		Same leg	Opposite leg
125	25	96	92
25	100	81	68
15	75	72	53
15 (8x)	25	92	88

Even when penicillin was administered before inoculation with *Cl. welchii*, the early inflammation, swelling, and discoloration which resulted in the local lesion was observed. Only a small lesion was produced, however, if a large dose of penicillin was used, and if repeated injections of the drug were made into the site of infection, the lesion healed completely within a period of 3 weeks. Direct smears from the lesion showed many of the organisms to be in long chains or filaments instead of single rods.

A comparison of the effect of penicillin and sulfonamide therapy (Table IV) begun before, at the same time, and at various intervals after infection, indicates that penicillin is superior to the sulfonamides. With penicillin,

TABLE IV.
Comparison of Effect of Time on Sulfonamide and Penicillin Therapy—Single Dose.

Time	% survival		
	Sulfathiazole*	Sulfadiazine*	Penicillin†
3hrs before *welchii*	—	97	—
Same time	45	53	100
1 hr after *welchii*	20	38	92
2 " "	14	16	80
3 " "	12	8	30

* 50 mg dose. See reference 2.
† 250 units—50 animals, same time, 1 hr and 2 hrs after; 10 animals, 3 hrs after.

the sharp drop in the survival rate does not occur until treatment has been delayed 3 hours. Deaths in animals treated with penicillin usually occurred 48 or more hours after inoculation while in the untreated and sulfonamide-treated animals, death occurred most frequently within 24 hours.

A few guinea pigs infected and treated in the same way as the mice have responded equally well to therapy.

No drug toxicity was observed in any of the animals.

Conclusions. 1. Penicillin is far superior to sulfonamides in the treatment of *Cl. welchii* infection in mice. A single injection of 50 Florey units of penicillin administered subcutaneously at the time of intramuscular inoculation with *Cl. welchii* protects 98% of the infected animals. 2. Repeated small doses of penicillin give as good protection as single large doses. 3. Delay in the institution of therapy lowers the survival rate, but not appreciably unless the delay is over 3 hours. 4. Local lesions were completely healed within 3 weeks if penicillin was injected repeatedly into the site of infection. 5. Response to the treatment of this infection in guinea pigs seems to be as good as that in mice.

14186

Distribution of a Vitamin B_1 Destructive Enzyme in Fish.

H. F. Deutsch and A. D. Hasler. (Introduced by H. C. Bradley.)

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"Chastek paralysis," a polyneuritic condition in foxes, has been produced when certain raw fish are included in the diet. Green *et al.*[1] showed that it could be cured with thiamine and more recently[2,3] that the pathologic manifestations resembled those of Wernicke's disease.

The ability of carp tissues to destroy vitamin B_1 has been reported by various investigators. Of these Woolley,[4] Sealock *et al.*,[5] and Spitzer *et al.*[6] suggest or recognize the enzymatic nature of the destructive factor. The latter workers showed that the destruction took place within the feed mixture. Deutsch and Ott[7] reported that thiamine destruction by freshwater smelt is likewise of an enzymatic nature. Wolf[8] found that buckeye shiners and saltwater herring are active in this destruction. The "cold storage herring" reported by Alexander *et al.*[9] to destroy vitamin B_1, appears to be the saltwater herring *(Clupea harengus)*. When trout were fed a diet containing this fish, the pathology of the central nervous system was analogous to that usually encountered in avitaminosis B_1 in higher vertebrates.

The purpose of this investigation was to study the distribution of the enzyme in fresh and saltwater fish by assay of as many species as were readily available.

Procedure. Fresh whole fish* or fresh fish viscera were finely ground to give a homogeneous mixture. Two equal portions were weighed and one was heated at 100°C for 15 minutes to inactivate the vitamin B_1 destructive enzyme if present. Equal amounts of dried brewer's yeast were then added to the raw and the heated portions, sufficient to provide 420-450 γ of thiamine per 100 g of fish preparation. After thorough mixing, these preparations were spread out on a flat surface and air-dried at room temperature under forced ventilation. Under these conditions 4-5 hours elapsed after mixing before any appreciable drying took place and the total period of moist contact of fish and yeast was approximately 12 hours. Previous work had shown that this period was greatly in excess of that required for complete destruction of the added vitamin B_1 under these conditions when smelt *(Osmerus mordax)* were used.

After thorough trituration in a mortar, a portion of the fish-yeast mixture (1-2 g) was assayed for vitamin B_1 by the standard thiochrome method. The presence of the destructive enzyme was indicated by the disappearance of thiamine from the unheated portion; if a trace remained it was usually less than 5% of the original amount added.

Results. Typical assays are shown in Table I. In Table II are the results of the analyses for the distribution of the enzyme in the series of fish examined. The species analyzed are arranged in taxonomic order.

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2 *Ibid., J. Nutrition*, 1941, **21**, 243.

3 Evans, C. A., Carlson, W. E., and Green, R. G., *Am. J. Path.*, 1942, **18**, 79.

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8 Wolf, L. E., Fisheries Research Bull. No. 2, N. Y. State Cons. Dept., Jan., 1942.

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* Most of the species common to the Great Lakes region were furnished through the courtesy of Smith Bros. of Port Washington, Wis. The saltwater species were provided by the Atlantic Coast Fisheries Co. of New York.

TABLE I.

Fish assayed	γ B_1/ g unheated fish-yeast mixture	γ B_1/g heated fish-yeast mixture	Presence or absence of the enzyme
Freshwater herring *(Leucichthys artedi arcturus)*	17.3	17.6	—
,, ,, ,, ,, ,,	15.8	16.6	—
,, ,, ,, ,, ,,	18.4	17.9	—
Crappie *(Pomoxis nigro-maculatus)*	11.8	12.9	—
Sauger pike *(Stizostedion c. canadense)*	0	21.9	+

TABLE II.

Family	Name	Presence or absence of the enzyme
Lepisosteidæ	*Garpike *(Lepisostous osseus oxyurus)*	—
Ameidæ	*Dogfish *(Amia calva)*	—
Coregonidæ	*Lake Michigan chub *(Leucichthys* sp.)	—
	*Lake Superior herring *(Leucichthys artedi arcturus)*	—
	‡Whitefish *(Coregonus clupeaformis)*	+
	†Menomonee whitefish *(Prosopium cylindraceum quadrilaterale)*	+
Osmeridæ	*Freshwater smelt *(Osmerus mordax)*	+
Salmonidæ	†Lake trout *(Cristivomer n. namaycush)*	—
	*Rainbow trout *(Salmo gairdnerii irideus)*	—
	*Brown trout *(Salmo trutta fario)*	—
Cyprinidæ	†Carp *(Cyprinus carpio)*	+
	*Goldfish *(Carassius auratus)*	+
	*Creek chub *(Semotilus a. atromaculatus)*	+
	*Fathead minnow *(Pimephales p. promelas)*	+
	§Buckeye shiner *(Notropus atherinoides)*	+
Catostomidæ	*Sucker *(Catostomus c. commersonnii)*	+
Ameiuridæ	*Channel cat *(Ictalurus lacustris punctatus)*	+
	*Bullhead *(Ameiurus m. melas)*	+
Umbridæ	*Mud minnow *(Umbra limi)*	+
Esocidæ	*Pickerel *(Esox lucius)*	—
Serranidæ	†White bass *(Lepibema chrysops)*	+
Percidæ	†Wall-eyed pike *(Stizostedion v. vitreum)*	—
	*Perch *(Perca flavescens)*	—
	†Sauger pike *(Stizostedion c. canadense)*	+
Centrarchidæ	*Crappie *(Pomoxis nigro-maculatus)*	—
	*Largemouth bass *(Huro salmoides)*	—
	*Smallmouth bass *(Micropterus d. dolomieu)*	—
	*Pumpkinseed *(Lepomis gibbosus)*	—
	*Bluegill *(Lepomis m. macrochirus)*	—
	*Rock bass *(Ambloplites r. rupestris)*	—
Gadidæ	†Burbot *(Lota lota maculosa)*	+
	Saltwater Forms.	
	‡Cod *(Gadus morrhua)*	—
	‡Haddock *(Melanogrammus aeglefinus)*	—
Scrombridæ	*Mackerel *(Scomber scombrus)*	—
Merluciidæ	*Whiting *(Merluccius bilinearis)*	—
Pleuronectidæ	*Lemon sole *(Pseudopleuronectes americanus dignabilis)*	—
	*Yellow tails *(Limanda ferruginea)*	—
	*Black backs *(Pseudopleuronectes americanus)*	—
Scorpaenidæ	*Red fish *(Sebastes marinus)*	—
Hippoglossidæ	*Dabs (unknown species)	—
Cupeidæ	§Herring *(Clupea harengus)*	+

* Whole fish.
† Viscera.
‡ Eviscerated fish. The whitefish showed 65% destruction.
§ From observations of Wolf.[8]

Discussion. The ability to destroy thiamine was found to occur fairly frequently among freshwater fish, but not in any of the saltwater species analyzed.

Green *et al.*[10] reported the enzyme present in herring from Lakes Michigan and Superior and in northern pike, whereas our results were negative with these species. Wolf[8] found that incorporation of the buckeye shiner *(Notropus atherinoides)* into the diet of the trout resulted in a B_1 deficiency. Smelt *(Osmerus mordax)*, however, showed no evidence of thiamine destruction under these conditions. Deutsch and Ott[7] proved by chick and thiochrome assay that smelt contain the destructive enzyme. However, it is noteworthy that Wolf's results on the buckeye shiner (Cyprinidæ) are consistent with our observations on this family.

In 3 analyses of the herring reported in Table II we were unable to show thiamine destruction. In addition, mink feeds containing 20-30% lake herring *(Leucichthys* sp.) were allowed to stand 24-36 hours before being assayed for thiamine. No vitamin B_1 destruction occurred.

Wolf's observation[8] on the presence of the factor in Atlantic herring *(Clupea harengus)* is the first clear-cut evidence of thiamine destruction by a saltwater fish. Green *et al.*[10] reported that Atlantic whiting and Pacific mackerel contained this factor. Our analyses showed no thiamine destruction by the Atlantic whiting *(Merluccius bilinearis)* and mackerel *(Scomber scombrus)*. Except for Pacific mackerel it is probable that the species were the same, but this is not certain because they failed to give scientific names. Moreover, their report on both fresh and saltwater species appears to be based on observations at different fur farms where these fish were fed rather than on controlled experiments.

Investigators have reported various amounts of thiamine in the edible portions of certain market fish. Their failure to consider the possibility of an enzyme in the tissues, that destroys thiamine, is fairly good evidence that the following varieties do not possess this enzyme:

Fish*	B_1/100 g	Investigator
Cod (fresh)	90	Munsell[11]
Haddock (fresh)	42	"
Cod (muscle)	120	Baker and Wright[12]
Halibut (fried)	180	" " "
Whiting (raw)	90	" " "
Sardine (tinned)	90	" " "
Herring (raw muscle)	9-12	Harris and Wang[13]
Halibut (edible portion)	84	Booker and Hartzler[14]
Trout (edible portion)	87	" " "

*Scientific names were not given.

Carp *(Cyprinus carpio)* and smelt *(Osmerus mordax)* have the destructive factor, and when they have undergone ordinary marketing or analogous handling no thiamine is found in the viscera or in the edible portions. On the other hand, tissues which are quickly inactivated with heat after removal from living carp and whole smelt contain thiamine.[15]

The taxonomic correlation of the presence or absence of the factor within certain families of fish is not constant; moreover, neither food habits nor environment appear to offer a basis of prediction as to whether a fish does or does not possess this enzyme. An extensive investigation of the saltwater fish and of the invertebrates may help in establishing any such relationship, should one exist.

Conclusion. Use of the described procedure permits easy determination of the presence of a vitamin B_1 destructive enzyme in fish tissues. The distribution of this enzyme in 31 species of fish in the Great Lakes region has been studied and its occurrence is rather common. On the other hand, our results with 9 species of saltwater fish all proved negative.

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[15] Unpublished data.

14187

Studies of Pectin Administration to Patients Not in Shock.*

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Pectin solution as a plasma substitute has been recommended by Hartman *et al.*[1,2] They demonstrated its effectiveness in experimental shock and in the prevention and treatment of shock in man. It was furthermore shown that pectin raises the plasma volume.[3]

To evaluate the usefulness of pectin solution as a plasma substitute, we studied its ability to cause hemodilution, which is a basic requirement of a plasma substitute; in addition, any untoward results following its administration were looked for, especially pseudo-agglutination of the erythrocytes. The latter has been considered an important contra-indication to the use of plasma substitutes.[4,5]

For better comparison, this study was made on 20 convalescent patients not in shock. Eight patients received a 0.75% and 12 a 1.5% solution of pectin‡ in approximately 90 minutes. The following were determined before and after the administration of pectin: (1) Arterial and venous pressure, and (2)—on venous blood drawn without tourniquet and placed into test tubes containing heparin§—hemoglobin, hematocrit, total protein (by the falling drop method), albumin and globulin (by Nesslerization), non-protein nitrogen, and sedimentation rate. Furthermore, complete hematologic data were obtained from 6 patients before and after pectin administration.

For comparison with pectin, 12 patients received a few days before or after the pectin administration, 1,000 cc of 2.5% dextrose in 0.4% saline intravenously in a period of 90 minutes.

As seen in Table I, after pectin administration the hematocrit, hemoglobin, and the total protein concentration and N.P.N. of the plasma decreased consistently, hemoglobin to a lesser degree. An increase of the mean corpuscular hemoglobin concentration was found in many instances. The albumin-globulin ratio did not change significantly. Systolic and diastolic arterial pressure and venous pressure rose in most instances. The sedimentation rate which was not at a normal level in a number of cases at the beginning of the experiment, because of the underlying disease, rose invariably. All reported constants usually returned to normal within 24 hours, in a few instances in 48 hours. The sedimentation rate, however, was sometimes found markedly elevated, even after 48 hours. Hematologic examinations revealed rouleau formation in 5 out of 6 patients and was present up to 72 hours. No other significant hematological changes were found.

Fifteen of the patients had received, prior to and following the pectin administration,

* Supported by a grant from Frederick Stearns and Company, Detroit, Mich.

† Abbott Fellow, Department of Surgery, Northwestern University Medical School.

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‡ The pectin solution was obtained from Frederick Stearns and Company and is described as having a molecular weight of between 40-60,000, the 1.5% solution in Ringers' having an oncotic pressure slightly greater than that of human plasma and a viscosity less than that of whole blood. Before administration, the acid solution was buffered with sodium phosphate or sodium lactate to a pH of 7.2.

§ The heparin used was provided by Hoffman, LaRoche Company (Liquaemin).

TABLE I.
Results of Intravenous Administration of Pectin (20 Patients) and of Dextrose and Saline (12 Patients).

Determination	Preliminary (avg)	% of change following administration of 1000 cc of pectin	% of change following administration of 1000 cc dextrose (2.5%) and saline (0.4%)
Total protein (g %)*	7.18	—12.9	— 5.1
A/G ratio	1.41	0	0
Hematocrit, %	43.8	— 9.8	— 4.2
Hemoglobin, g	14.0	— 5.0	
Red cell count	4.45		+ 1.7
Sedimentation rate, mm/hr	53.2	+46.6	+ 6.1
Venous pressure, cm	12.2	+22.1	+15.5
Arterial blood pressure:			
Systolic	123	+14.8	
Diastolic	78.8	+19.6	

* Determined by Barbour-Hamilton Falling Drop Apparatus.

Evans Blue (T-1824) in total doses of as much as 90 mg intravenously without demonstrating the hemorrhagic diathesis reported by others.[3] Hematologic studies failed to reveal such complications.

In none of the cases were reactions observed following the intravenous pectin injections. There was no rise of temperature, no significant change in pulse rate, fever or chills.

The urine output during the pectin (1.5%) administration increased in 8 cases by 500 to 600 cc without a consistent change in protein or nitrogen excretion.

In the patients receiving dextrose and saline solution the total protein and hematocrit were only slightly reduced at the end of 90 minutes; the sedimentation rate rose slightly and the venous pressure moderately.

Discussion. From these studies it appears that pectin solution in the form given is an effective hemodiluting agent in patients not in shock, producing slight rise of arterial and venous pressures. Its hemodiluting effect is much more marked and prolonged than that of dextrose and saline solution and is more marked in the hematocrit than in the hemoglobin determination, which suggests the possibility that part of the reduction of the hematocrit is not true hemodilution but shrinkage of the red cells. The increase in the sedimentation rate due to rouleau formation caused by pectin solution is marked but the resulting sedimentation velocity is not in excess of that commonly found in infections. Whether that represents a contraindication to the use of pectin in the form given must be decided by further studies. Other untoward results were not encountered.

Similar studies using pectin solution in patients in shock are in progress and will be reported separately.

Conclusion. Pectin solution is an effective hemodiluting agent in patients not in shock. It lowers total plasma protein, hematocrit, hemoglobin, and plasma non-protein-nitrogen. It raises the venous and arterial pressure slightly and the sedimentation rate of the erythrocytes markedly.

14188

Fluorescent Granules at the Glomerular Pole of Human Kidneys.*

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An agglomeration of granulated cells at the glomerular pole of the kidney has been described[1,2,3] close to the part of the distal convoluted tubule with densely arranged cells (macula densa).[4] The granulated cells, originally compared with "epitheloid" cells of arteriovenous anastomoses,[5] were called neuromyoarterial juxtaglomerular apparatus by Goormaghtigh.[6] Observing hypertrophy and increased granularity of these cells in experimental renal hypertension, he[7] considered them as the source of the pressor substance, renin. Studies[8,9] on hypertensive rabbits supported this idea. Similar cells—apparently transformed smooth muscle cells—but without granules, were also seen in man; in hypertension they appeared hypertrophic.[10] Recent investigations,[11] however, indicate that renin is produced in the renal tubules.

The possible significance of the juxtaglomerular apparatus justifies description of new morphologic findings in this area. After short fixation in formalin, frozen sections of human kidneys were examined under the fluorescence microscope. Sometimes a sharply circumscribed agglomeration of fine granules with whitish to gold brown, not fading, fluorescence was seen at the glomerular pole (Fig. 1B). The granules surround or even invade the wall of the arteriole—recognized by blue fluorescent fibers—at its entrance into the glomerulus. A few granules may be found farther along the arteriole outside or inside the glomerulus. In other glomeruli the granules are seen on one side of the arteriole only, in a circumscribed ovoid area. The fluorescence was not changed by oxidizing or reducing agents, acid or alkali. Lipoid solvents reduced the fluorescence only after prolonged treatment; after paraffin embedding it was lost.

For exact localization of the fluorescent granules, sections were slightly stained with hemalum. This does not interfere with the fluorescence but allows observation of the same area in visible light. Thus the granules were found in the same area where in visible light the juxtaglomerular apparatus was seen, the outer limits of fluorescence and apparatus being identical. If a macula densa was visible, the granules extended up to it (Fig. 1A).

Kidneys of different individuals (107 cases) showed great variations in number, size, and fluorescence of the granules which were uniform in glomeruli of the same kidney. Their presence and number were independent of the development of the juxtaglomerular apparatus. Sometimes the granules were absent from a large apparatus. The fluorescent granules, absent in children, increased in number with age. They were numerous and relatively large in general exhaustion with brown atrophy of the organs. The fluorescence was usually striking in hypertension without renal involvement but reduced with renal involvement. In chronic nephritis it was reduced or absent. In hyalinizing glomeruli, the fluorescence may

* This work was supported by a grant from the Committee on Scientific Research of the American Medical Association.

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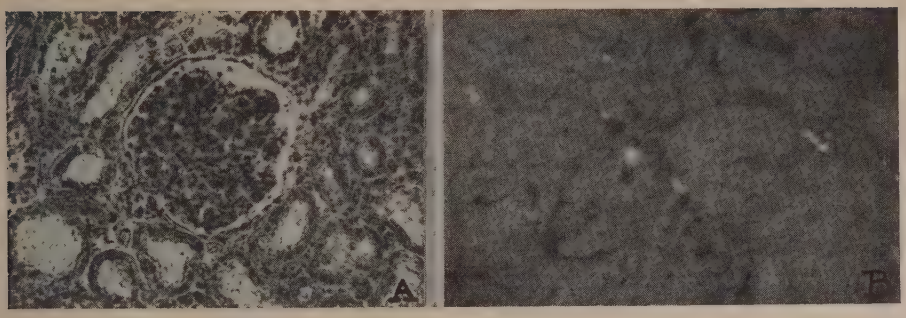

FIG. 1.

Juxtaglomerular apparatus of human kidney: (A) In visible light, agglomeration of cells close to macula densa (part of distal convoluted tubule with densely arranged nuclei). (B) In ultra-violet light, fluorescent granules on the glomerular pole.

remain in characteristic localization even if the nuclei of the juxtaglomerular apparatus have disappeared.

Besides these granules the following fluorescent details are occasionally found in human kidneys: irregularly formed large particles in scars, sclerotizing or hyalinized glomerularized or arteriosclerotic vessels; ultra-violet labile vitamin A fluorescence of lipoids in pathologic conditions;[12] and brown fluorescent granules in tubular epithelium. The fluorescence of these latter two is of different character.

In kidneys of monkeys, dogs, cats, mice, rats and rabbits no fluorescence was found at the glomerular pole. It was absent in dogs with experimental renal hypertension.†

The fluorescent granules are not a normal characteristic of the juxtaglomerular apparatus and not related to the granules seen in visible light in animals. Age distribution, solubility, localization in modified smooth muscle cells, and character of fluorescence[13] justify their interpretation as wear and tear pigment. Their deposition into the apparatus is probably a common process, comparable to lipoid deposition in arteriosclerosis. The fluorescence is not related to renin since renin preparations showed no characteristic fluorescence.‡

Conclusions. Fluorescent granules are sometimes found at the glomerular pole of human kidneys. They are absent in normal animals and in dogs with experimental renal hypertension.

[12] Popper, H., *Arch. Pathol.*, 1941, **31**, 766.

† Thanks are due to Dr. H. Goldblatt and Dr. G. E. Wakerlin for kidney specimens of dogs with experimental renal hypertension.

[13] Hamperl, H., *Virch. Arch. f. path. Anat.*, 1934, **292**, 1.

‡ We wish to thank Dr. G. E. Wakerlin for the renin preparations.

14189 P

Effect of Intracisternal Injection of Potassium Phosphate in Hemorrhagic Hypotension and Shock in the Dog.

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According to Stern[1] the injection of small amounts of potassium phosphates into the cisterna magna restores an animal from shock, even when due to loss of blood. The resulting elevation of arterial pressure is attributed to direct stimulation of the sympathetics by the potassium and to decrease in the tonus of the parasympathetics through the decrease in ionization of calcium by the phosphate ion. She also reports favorable results from the cisternal injection of 1-2 cc of the solution on the battlefield. We are reporting herewith the results we have obtained with intracisternal injection into dogs of the potassium phosphate solution employed by Stern.

Seven dogs under intravenous barbital anesthesia were subjected to varying degrees of hemorrhage from the femoral artery. Mean blood pressure was recorded with the mercury manometer from the other femoral artery, and the respirations by thoracic cage tambour. The drawn blood was reinjected immediately and followed by injection of 1/6 molar (isotonic) solution of potassium phosphate (mixture of KH_2PO_4 and K_2HPO_4 brought to pH 7.6).[2,3] by cisternal needle, and with the dog's head in a lateral dependent position. On the basis of 2 cc for a 70 to 75 kilo man, 0.4 to 0.5 cc was considered a therapeutic dose for the 15 to 18 kilo dog.

Injections of the solution at levels of arterial pressure greater than 60 mm of Hg. produced in one unbled and in 3 previously bled and transfused dogs a preliminary inspiratory pause followed by a rapid increase in rate and amplitude of respirations. There was a significant increase in blood pressure, as great as 90 mm, with initial increase in heart rate followed by slowing. The rise in blood pressure was maintained.

In dogs subjected to repeated hemorrhages varying from 10 to 60% of the estimated blood volume and with hypotension levels of less than 50 mm maintained for 1 hour and in others in which the arterial pressure was maintained at levels of 30 mm for 30 to 45 minutes, the responses were not uniform. In 3 instances respirations were actually depressed following the injection of the drawn blood and 0.4 cc of the solution, and the dogs died within 2 to 3 minutes with rapid fall in arterial pressure. In all cases respiration was arrested before any change in pulse or blood pressure was produced. When the blood and salt (0.4 cc) were injected immediately following cessation of respiration due to shock there was no recovery. This occurred even though artificial respiration was maintained and the heart beat was vigorous and the mean arterial pressure between 40 and 70 mm Hg. In one instance there was a slight rise in blood pressure which may have been due to asphyxia.

In 3 instances (one unbled dog and 2 bled and transfused dogs with arterial pressures of 90 mm or more), injections approximating 4 times the calculated therapeutic dose were followed by marked stimulation of respiration and rise of blood pressure which was maintained for 6 to 10 minutes, following which the arterial pressure fell abruptly, respiration became irregular and death ensued.

Summary. Although the intracisternal injection into normal dogs of therapeutic doses of potassium phosphate increases the rate and amplitude of respiration, the arterial pressure and heart rate, the response in hemorrhagic hypotension or shock was not uniform. Injection into normal dogs of larger doses resulted in death.

1 Stern, L. S., *Schweiz. Med. Wohnschr.*, 1941, **71**, 367.

2 Stern, L. S., *Lancet*, 1942, **243**, 572.

3 Stern, L. S., *Brit. Med. J.*, 1942, **2**, 538.

14190

Comparison of Chlorine and Ozone as Virucidal Agents of Poliomyelitis Virus.*

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Levaditi, Kling and Lépine,[1] upon finding that 0.4 ppm of chlorine inactivated poliomyelitis in 24 hours, assumed that chlorination of water by the methods usually employed for purification of drinking water, was adequate for destruction of the virus.

As a result of numerous recent observations[2-7] in which poliomyelitis virus has been recovered from sewage, from stools of active cases and from stools of carriers, Kempf and Soule[8,9] reopened investigations on the subject of the virucidal effects of chlorine on this virus. They found that concentrations of 0.5 ppm of chlorine failed to inactivate the MV strain of poliomyelitis virus in 1:1650 dilution in 1½ hours at a temperature of 21-24°C and a pH of 8.5. Trask and Paul[10] likewise report that one strain with which they worked was resistant to chlorine.

Studies in this laboratory[11] on the comparative germicidal effects of chlorine and ozone on cysts of *Endamoeba histolytica* and on certain bacteria, showed ozone to be the more active agent. It was, therefore, decided to compare ozone and chlorine as virucidal agents of poliomyelitis virus.

Materials and Methods. Two strains of virus were used during the experiments. The MV, an extensively used experimental strain, was employed in the first experiment and Le, a strain recently isolated in Los Angeles, in the second and third experiments. At the time of the experiments, the pool of MV virus used produced symptoms in 100% of the animals inoculated at a titer of 10^{-1} while the pool of Le virus did the same at a titer of 10^{-3}.

The virus to be tested was diluted in saline solution, placed in test tubes and treated as follows:

1. *Chlorine.* In the chlorination tests each tube contained 15 cc of virus and chlorine consisting of 13½ cc of virus and 1½ cc of sodium hypochlorite or of gaseous chlorine solution of sufficient strength to give the desired terminal residual. Tubes were corked and agitated immediately upon mixing. They were shaken at 2-minute intervals during the period of the experiment. Each tube constituted a unit for each time period to be appraised, pH and residual values being determined at the end of each period. Tubes were kept in a constant temperature bath during the experiment.

2. *Ozone.* Ozonized air was bubbled through 50 cc of the mixture to be tested, a suitable diffusor carrying the ozone to the bottom of the tube. Amounts of virus mixture necessary to test pH and residual ozone and for inoculation into animals were removed at the required

* Supported by grants from the National Foundation for Infantile Paralysis, Inc., and from Lane-Wells Company.

1 Levaditi, C., Kling, C., and Lépine, P., *Bull. Acad. de Méd.*, Paris, 1931, **105**, 190.

2 Paul, John R., Trask, J. P., and Culotta, C. S., *Science*, 1939, **90**, 258.

3 Trask, James D., Paul, John R., and Vignec, A. J., *J. Exp. Med.*, 1940, **71**, 751.

4 Howe, Howard A., and Bodian, David, *J. Infect. Dis.*, 1940, **66**, 198.

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6 Piszczek, E. A., Shaughnessy, H. J., Zichis, J., and Levinson, S. O., *J. A. M. A.*, 1941, **117**, 1962.

7 Kessel, John F., Moore, Frederick J., Stimpert, F. D., and Fisk, R. T., *J. Exp. Med.*, 1941, **74**, 601.

8 Kempf, J. Emerson, and Soule, Malcolm H., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 431.

9 Kempf, J. Emerson, Wilson, Martha F., Pierce, Marjorie E., and Soule, Malcolm H., *Am. J. Pub. Health*, 1942, **32**, 1366.

10 Trask, James D., and Paul, John R., *Am. J. Pub. Health*, 1941, **31**, 239.

11 Kessel, John F., Quiros, Maria, Allison, Donald K., and Kaime, M., manuscript.

TABLE I.
Summary of Inoculation Experiments. Initial pH 6.9. Temperature 20°C.

Exposure times	7½ min	15 min	30 min	60 min	120 min
Exp. No. 1, MV virus 1:10. Sept. 30, '42					
Ozone*	2/2†	2/0	1/2	—	—
Controls					8/8
Exp. No. 2, Le virus 1:1000. Nov. 18, '42					
Ozone*	0/2	0/2	0/2	0/2	0/2
Controls					2/2

Exp. No. 3, Le virus. Dec. 27, '42 Exposure times	2 min	5 min	15 min	45 min	90 min	180 min.
Controls, virus 1:1000						
Without thiosulfate						3/3
With thiosulfate						3/3
Ozone—Residual 0.05—0.45 ppm						
Virus 1:10			3/3	1/3		
" 1:100			1/3	0/3		
" 1:1000	0/3	0/3	0/3	0/3		
Chlorine—Virus 1:1000						
Gaseous						
Residual 0.25-0.3 ppm			2/3			0/3
" 0.5-1.0 "		2/3	1/3	1/3	1/3	0/3
Sodium hypochlorite						
Residual 0.5-1.0 ppm			2/3	1/3	1/3	0/3

* Residual of ozone in ppm did not exceed 0.2 in any test.
† Numerator—Animals developing paralytic poliomyelitis. Denominator—No. of animals inoculated.

intervals, at which time pH and residual values were determined.

The chlorinated and ozonized virus mixtures to be injected into monkeys were treated with identical amounts of sodium thiosulfate immediately upon removal from the test mixtures. One cc of the treated virus was injected intracerebrally into each test animal *(Macaca mulatta)* within 10 minutes after neutralization.

The ozone used was produced by a laboratory model of the Sterozone ozonizer, designed by one of the authors, D.K.A. Ozone produced by this method contains negligible amounts of nitrogen oxide as determined by the diphenyl benzidine test. The orthotoluidine method was used to determine the residual in ppm, both for ozone and chlorine. The pH determinations were made electrometrically.

Conclusions and Summary. 1. Gaseous chlorine and hypochlorite in residual amounts of 0.5 ppm failed to inactivate a 1:1000 dilution of Le strain of poliomyelitis virus after an exposure of 90 minutes but inactivated the virus by 180 minutes. These results compare favorably with those of Kempf and Soule in working with the MV strain of virus. 2. Ozone in residual amounts not exceeding 0.45 ppm, inactivated a 1:1000 dilution of Le virus in 2 minutes and a 1:100 dilution of the same virus in 45 minutes. A 1:10 dilution of MV virus was not inactivated in 30 minutes and a 1:10 dilution of Le virus was not inactivated in 45 minutes. The extra amount of brain material in the lower dilutions constitutes an excess of organic matter which probably accounts for the longer time necessary for inactivation of virus at these dilutions.

It is observed from these results that an identical dilution of the same strain and pool of poliomyelitis virus when exposed to chlorine in residual amounts ranging from 0.5-1.0 ppm and to ozone in residual amounts between 0.05-0.45 ppm under the same controlled experimental conditions was inactivated almost immediately by ozone, *i.e.*, within 2 minutes, while an interval ranging between 1½ hours and 3 hours was required for inactivation by chlorine.

Thompson[12] in a recent review of the significance of the findings concerning the occurrence of poliomyelitis virus in intestinal con-

[12] Thompson, Rudolph E., *Water and Sewage*, 1943, **81**, 24.

tents and sewage and of its inactivation by current water purification procedures suggests either "superchlorination" or that "ozone treatment might be effective."

The germicidal effects of ozone have been noted in the past but recent quantitative determinations have not been reported and its effect on viruses has not been observed. The current results indicate that ozone acts as a strong virucidal agent and warrant additional experimentation.

14191

Studies on the Nutrition and Metabolism of *Pasteurella pestis.*

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Although *Pasteurella pestis* has been the subject of a great deal of medical and immunological research, very little work has been done on the physiology of the organism itself. During recent years, attempts have been made to apply technics used in the study of bacterial metabolism to certain pathogenic organisms. The experiments reported in this paper were designed to establish the basic growth requirements of the plague bacillus, and to investigate to some extent the nature of its dissimilatory processes.

Rao[1] claims that 3 amino acids, cystine, phenylalanine, and proline, are essential for the development of *P. pestis,* and that haematin and riboflavin may be considered as accessory growth factors. However, he does not give evidence that the organism can grow with a mixture of these compounds alone, and provides neither a carbon nor a nitrogen source other than amino acids in most of his media. In an excellent study dealing with the nutrition of the members of the genus *Pasteurella,* Berkman[2] could find no effect of 12 nutrilites on the development of their strains of *P. pestis* in a medium containing glucose as well as a mixture of amino acids.

Experiments were carried out in this laboratory and at the Hooper Foundation for Medical Research with a non-pathogenic strain (1122) of *P. pestis,* and with 3 selected pathogenic strains: 337 (from India), and "Yreka" and "Shasta" from California. Since glucose was found to be the best carbon source and ammonium salts an excellent nitrogen source for all organisms tested, the basic medium was prepared with 0.2% glucose, 0.1% NH_4Cl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.005% $FeCl_3$, .001% $CaCl_2$, and M/30 Sorensen KH_2PO_4-Na_2HPO_4 buffer at pH 7.0. Five ml amounts of the basic medium were dispensed in 17 mm test tubes to insure a fair degree of aeration without agitation. All cultures were incubated at 29°C. On the first transfer from blood agar slants, none of the strains tested could grow in the above medium unless small amounts (0.002%) of cystine and phenylalanine were added. Initially large inocula had to be used, and visible turbidity was slow to appear, evidence pointing to a selection of the cells most capable of reproducing in this environment.[3] With subculturing in the same medium, growth became rapid. The addition of 0.002% proline hastened the development of strains 337 and "Shasta" on first transfer, but had no appreciable effect on their subsequent growth. The other strains tested were unaffected by proline. No nutrilite function of haematin, biotin, pantothenic acid, *p*-aminobenzoic acid, riboflavin, nicotinic acid, thiamin, or pyridoxine could be detected for any of the strains.

More detailed studies carried out with strain 1122 showed that:

1. Cystine could be replaced with thiosulfate, sulfite, thioglycollate, or homocystine, but not with methionine.

[1] Rao, M. S., *Indian J. M. Research,* 1939, **27**, 75.

[2] Berkman, S., *J. Infect. Dis.,* 1942, **71**, 201.

[3] Doudoroff, M., *J. Bact.,* 1942, **44**, 451.

2. Phenylalanine normally supported maximum development at a concentration of 0.0002%, but the bacteria could be "weaned" from this requirement to develop at almost their normal rate without it. Strains adapted in this manner could accept glucose as sole carbon source with thiosulfate for source of sulfur. Substitution of tyrosine, and, to a lesser extent, of tryptophane for phenylalanine was found helpful, but not necessary, in effecting the "weaning" (*c.f.* role of homocystine in a similar phenomenon observed with luminous bacteria.[3]) Neither tyrosine nor tryptophane appeared to be as satisfactory to the bacteria as phenylalanine, and it was found impossible to use phenylacetic and indoleacetic acids as substitutes.

3. With cystine and phenylalanine added to the medium, mannitol was found to be almost as good a carbon source as glucose. Pyruvate and lactate were fair, alanine and proline poor, while glycerol, ethanol, succinate, malate, and asparagine were entirely unsatisfactory.

4. Amino acids were in general no more satisfactory as nitrogen sources than were ammonium salts. The addition of glycine to the basic medium had no growth-promoting effect, as claimed by Rao.[1] Although the bacteria could reduce nitrate to nitrite, they appeared to be unable to use the former as a nitrogen source.

5. Certain amino acids were inhibitory to growth when added to simplified media. Thus, the addition of 0.002%-0.01% *l*- or *dl*-leucine to the basic medium with either cystine or thiosulfate caused a prolonged lag in the development of strains adapted to these media without leucine. This effect could be reduced or eliminated by the addition of isoleucine or valine, neither of which appeared to have any growth-promoting value by itself. Methionine was found to be inhibitory when cystine was used as sulfur source, but not when thiosulfate was used. The interaction of amino acids in growth plays havoc with the commonly used "elimination method" of determining minimal requirements, since often the elimination of one constituent prevents development, not because of its indispensability to the organism, but because of its value in counteracting the toxic effect of another substance in the mixture.

6. Very heavy cultures of strain 1122 could be obtained in synthetic media with glucose and small amounts of cystine and phenylalanine if adequate aeration was provided by constant agitation. Pyruvic acid was found to accumulate in the course of development (to about 0.01 N) and to disappear after the sugar had been oxidized. In unagitated cultures, the bacteria died rather rapidly because of the accumulation of pyruvic acid and possibly of fermentation products as well. Pyruvic acid was also produced in cultures of this strain with mannitol as chief carbon source and by all of the other strains of *P. pestis* tested in glucose-containing medium. Pyruvic acid was identified by the preparation of its 2,4-dinitro-phenylhydrazone. (Melting points uncorrected; hydrazone of pyruvic acid, 214.0°C; unknown, 213.25°C; mixed, 213.5°C.) Quantitative estimation was made by the method of Elliot and c.w.[4]

TABLE I.
Fermentation of Glucose by *Pasteurella pestis*.

	Per 100 cc of medium		
	Millimoles	Milliatoms C	Milliatoms "available H" (ref. 5)
Glucose utilized	1.810	10.860	43.44
CO_2	0.247	0.247	—
Formic acid	0.343	0.343	0.69
Acetic acid	1.747	3.494	13.98
Ethanol	1.010	2.020	12.12
Lactic acid	1.027	3.081	12.32
Pyruvic acid	0.025	0.075	0.25
Succinic acid	0.284	1.136	3.98
Products recovered		10.396	43.34
% of recovery		95.8%	99.8%

7. Strain 1122 was capable of development under completely anaerobic conditions in complex media containing glucose. No attempts were made to analyze growth requirements under such conditions. The chief products of glucose fermentation appeared to be lactic and acetic acids and ethyl alcohol. Carbon dioxide, formic acid, succinic acid, and a small amount of pyruvic acid were also found. No hydro-

[4] Elliot, K. A. C., Benoy, M. P., and Baker, Z., *Biochem. J.*, 1935, **29**, 1937.

gen, glycerol, or 2,3-butylene glycol could be detected, and only traces of acetyl methyl carbinol or diacetyl could be shown. A fair carbon and hydrogen balance[5] was obtained for the fermentation of glucose in a medium initially containing 326 mg glucose, 45 mg Difco yeast extract, 3 mg proline, 3 mg phenylalanine, 1.5 mg cystine, 1 mg isoleucine, 1 mg valine, 45 mg NH_4Cl, 45 mg $MgSO_4 \cdot 7\ H_2O$, 2 mg $CaCl_2$, and 1 mg $FeCl_3$ per 100 cc of 0.05 M Sorensen phosphate buffer at pH 7.5. During the fermentation the medium was neutralized at intervals with NaOH, until all of the sugar had disappeared. The results are presented in Table I.

Although the carbon recovery appears to be satisfactory, the ratio of two-carbon to one-carbon derivatives (see 5) is extraordinarily high for an ordinary fermentation, being approximately 3.2. This might indicate that a portion of the one-carbon derivatives remains unaccounted for (as unidentified products in the medium or in the bacterial) or, perhaps that part of the 2-carbon compounds may arise, not through the splitting of a 3-carbon degradation product of sugar, but by some other method such as the reduction of carbon dioxide.

5 Doudoroff, M., *J. Bact.*, 1942, **44**, 461.

14192

Resistance of Small Colony Variants (G-Forms) of a Staphylococcus Towards the Bacteriostatic Activity of Penicillin.

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So far, two types of penicillin-resistant staphylococci have been described:

(1) Naturally resistant strains, as reported by Fleming,[1] and by Hobby, Meyer and Chaffee.[2] We, too, found 5 resistant strains of *Staphylococcus aureus* in a group of 12.

(2) Strains rendered drug-resistant by prolonged contact of the organisms with increasing concentrations of penicillin (Abraham and co-workers,[3] Smith and Hay,[4] Rammelkamp and Maxon.[5]) These strains may exhibit slight morphological changes, such as larger size of the coccus[4] or a reduced velocity of growth and enzyme activity.[3]

A third type of penicillin-resistant staphylococci, which has not been described before, is represented by variants occurring when a normal strain of *Staphylococcus albus* was exposed to penicillin. Such strains were studied in our laboratory during recent months. They show the profound cultural changes known as small colony variants (Hoffstadt and Youmans,[6] Swingle,[7] Youmans,[8] Youmans and Delves.[9])

Material and Methods. The penicillin used was produced by growing a strain of *Penicillium notatum* No. 1209* in a modified

1 Fleming, L., *Lancet*, 1942, **1**, 732.

2 Hobby, G. L., Meyer, K., and Chaffee, E., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 277.

3 Abraham, E. P., Chain, E., Fletcher, C. M., Florey, H. W., Gardner, A. D., Heatley, N. G., and Jennings, A. M., *Lancet*, 1941, **2**, 177.

4 Smith, L. D., and Hay, T., *J. Franklin Instit.*, 1942, **233**, 598.

5 Rammelkamp, C. H., and Maxon, T., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 386.

6 Hoffstadt, R. E., and Youmans, G. P., *J. Infect. Dis.*, 1932, **51**, 216.

7 Swingle, E. L., *J. Bact.*, 1935, **29**, 467.

8 Youmans, G. P., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 94.

9 Youmans, G. P., and Delves, E., *J. Bact.*, 1942, **44**, 127.

* We are indebted to Dr. Robert D. Coghill, Bureau of Agricultural Chemistry and Engineering, Northern Regional Research Laboratory, Peoria, Ill., for the strain of *Penicillium notatum* and of *Staphylococcus albus* No. 314.

Czapek-Dox medium.[3] Initial experiments were conducted with Seitz filtrates of the crude culture fluids, containing 4-8 Oxford units per ml, while the later work was carried out with concentrates of barium penicillate containing 500 Oxford units per ml.† The values given for penicillin are expressed in Oxford units as defined by Florey and Jennings.[10]

All experiments were conducted with a strain of *Staphylococcus albus* No. 314* as the test bacterium. The media for culturing the Staphylococcus and for all the tests carried out in these studies were papain digest broth and agar prepared according to the method of Asheshov.[11]

The ring test (Abraham and co-workers[3]) and the dilution method in liquid media were used for measuring the sensitivity of our strains. The dilution or titration method was carried out in test tubes containing serial dilutions of penicillin in a volume of 5 ml. The tubes were seeded with 0.1 ml of a 20-hour broth culture and the results read after 18 to 20 hours incubation. *In vitro* tests with methyl violet were carried out similarly but the volume was only 2 ml.

Experimental. In a ring test with *Staphylococcus albus* No. 314 a crude culture fluid of *Penicillium notatum* produced an area of inhibition of 25.5 mm diameter. This area was covered almost completely with a thin film of minute translucent colonies of staphylococci. This growth was obviously identical with the ghost-like "halos of partial inhibition" mentioned briefly but not further investigated by Abraham and co-workers.[3] By subculturing some of the minute colonies a fair growth of these very small colonies was obtained. They exhibited the morphological and cultural characteristics of small colony variants described by former investigators.[6,7,8,9] Two days later a few colonies of normal appearance were found in subcultures but the small colonies could be isolated again and propagated on liquid and solid media. This strain of small colony variants (No. 314 G) was then split up in 3 cultures which were transferred daily on agar plates for 20 days. One culture was kept on plain papain digest agar (No. 314 G a), the other on the same agar base containing 10% of a Seitz filtrate of culture fluid of *Penicillium notatum* (No. 314 G b), and the third culture (No. 314 G c) was kept on agar containing 1% of the same culture fluid. The strains grew abundantly on these media, without any reversion to the normal growth. If kept on agar plates and slants with only one weekly transfer to fresh medium, the 3 strains were stable. During 4 months no reversion was observed. However, if daily transfers were made on rich media, such as beef heart infusion and yeast extract broth and agar, all 3 G-forms reverted to the normal type of growth within one week.‡

The most interesting property of the small colony variants was their resistance to penicillin. In frequent ring tests carried out in triplicate the parent strain showed consistently normal sensitivity with an area of inhibition of 19 to 25 mm. Small colony variants were not inhibited at all, while reverted strains regained the sensitivity of the parent strain. There was no difference in penicillin resistance between the strains No. 314 G a, No. 314 G b, and No. 314 G c, indicating that the single initial exposure to penicillin had sufficed to produce a stable penicillin-resistant G-form.

In order to find out whether the resistance was specific, *i.e.*, due to the primary exposure to the bacteriostatic agent, the modified strains produced by penicillin and variants obtained by exposure to $BaCl_2$[8,9] were compared with respect to their sensitivity to penicillin concentrate and to methyl violet.

By growing Staphylococcus No. 314 in papain digest broth containing 2%, 1%, 0.5% $BaCl_2$, abundant formation of G-forms occurred after 48 hours incubation. Three strains, marked No. 314 G B 2, No. 314 G B 1, and No. 314 G B 05, were isolated from the 3 $BaCl_2$ media. They yielded typical small colony variants; Strain No. 314 G B 05,

† Prepared by Drs. Goldberg, Oppenheim-Errera (deceased), and Scott of the Roche Laboratories.

10 Florey, H. W., and Jennings, M. A., *Brit. J. Exp. Path.*, 1942, **23**, 120.

11 Asheshov, I. N., *Canad. Pub. Health J.*, 1941, **32**, 468.

‡ Observations made by B. Tabenkin of the Roche Laboratories, who also supplied us with the crude culture fluids of *P. notatum*. Grateful acknowledgment is made of his cooperation.

TABLE I.
Sensitivity of Staphylococcus No. 314 and Its Small Colony Variants to Penicillin and Methyl Violet.

Strain	Variants produced by exposure to	Inhibition by penicillin: Dilution method Oxford units/ml	Inhibition by penicillin: Gutter plate, mm*	Inhibition by methyl violet
No. 314	—	0.002	19-19.5	1-1280 × 1000 -2560 "
" G a	Penicillin	>1	0	1-320 "
" G b	"	>1	0	1-320 "
" G c	"	>1	7.5	1-320 "
" G B 2	$BaCl_2$	—†	—	1-320 "
" G B 1	"	0.125	5	1-320 "
" G B 05	"	0.5	0	1-320 "
" G B 2 reverted	"	—	20	—

* Distance from the edge of the gutter to the zone of full growth.
† The strain was lost at the time of these experiments.

derived from the medium with 0.5% $BaCl_2$, was the only one which showed less translucent colonies with a slightly whitish surface. The strains were fairly stable if overnight cultures on agar slants were kept at room temperature and not transferred more frequently than once a month.

In Table I the results of the *in vitro* experiments with the original strain and its different variants are tabulated. The penicillin used in these experiments was a concentrate of barium penicillate containing 500 Oxford units per ml. The agar strip of the gutter plate (Fleming[1]) was prepared with an aqueous solution of barium penicillate and contained 30 Oxford units per ml.

The table demonstrates the greatly increased resistance of the small colony variants in comparison with the normal sensitivity of the original strain and of one of the reverted cultures. Moreover, all small colony variants show a markedly reduced sensitivity towards the bacteriostatic activity of methyl violet.

Discussion. The observations presented in the present paper indicate that the small colony variants of a strain of *Staphylococcus albus* are resistant to penicillin. Although strains obtained by previous exposure to penicillin appear slightly more resistant than the strains produced by exposure to $BaCl_2$, these differences can hardly be considered significant. This resistance to penicillin is not a specific drug-fastness, but seems to be part of the profound modification undergone by these dissociated strains. The reduced sensitivity of the small colony variants to a completely different chemical agent, such as methyl violet, seems to substantiate this view and indicates that these variants acquired a non-specific resistance towards specific antibacterial agents. This is a phenomenon similar to the resistance of small colony variants of staphylococci to staphylococcus bacteriophage as described by Hoffstadt and Almaden.[12] It is interesting to note that Philipps and Barnes[13] encountered 6 different forms of variants when they produced resistance of *Staphylococcus aureus* towards gramicidin. By eliminating the variants, they obtained gramicidin-resistant strains with otherwise normal cultural appearance, enzyme activity, and unchanged sensitivity to crystal violet. Strains like these probably belong in the same group as the tyrothricin-resistant staphylococci described by Rammelkamp[14] and the penicillin-resistant strains of Abraham and co-workers[3] and Rammelkamp and Maxon[5], and may represent examples of genuine and specific drug-fastness. The resistance exhibited by our strains of small colony variants is not specific.

Summary. 1. Small colony variants (G-forms) of a strain of *Staphylococcus albus* were obtained on agar by the action of penicillin. 2. These variants showed a high

[12] Hoffstadt, R. E., and Almaden, P., *J. Infect. Dis.*, 1934, **54**, 253.

[13] Philipps, R. L., and Barnes, L. H., *J. Franklin Instit.*, 1942, **233**, 396.

[14] Rammelkamp, C. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 346.

resistance towards the bacteriostatic activity of penicillin. 3. Small colony variants of the same strain of *Staphylococcus* produced by the exposure to $BaCl_2$ possessed likewise a considerable resistance to penicillin. 4. All these strains of small colony variants regardless of their origin acquired also a certain degree of resistance towards methyl violet. 5. The resistance of these strains of small colony variants towards penicillin cannot be considered to be a specific drug-resistance.

14193

Effect of Neosynephrin* on Gaseous Exchange of the Brain.†

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The effects of neosynephrin on the cerebral arterio-venous oxygen difference were studied on large dogs, narcotized with Dial,‡ 0.5 cc per kilo. The cranium was trephined above the superior longitudinal sinus, the trachea was exposed and cannulated and both femoral arteries were also exposed. The first pair of femoral arterial and cerebral venous blood samples was collected while the animals were breathing air and the second after they had come to equilibrium while respiring a gas mixture containing approximately 11% oxygen. Neosynephrin was then administered and when blood pressure had risen significantly the third pair of blood samples was collected. Blood flow of the cerebral longitudinal sinus was determined by Daly's modification of the thermostromuhr.[1] Blood pressure was recorded by means of a cannula inserted into a femoral artery. Blood samples were analyzed for oxygen and carbon dioxide contents and oxygen capacity,[2] for glucose,[3] and for lactic acid.[4] The oxygen tension of the blood was estimated by using the nomogram of Bock *et al.* for humans[5] which may also be applied to dogs.[6] In addition, the effects of neosynephrin on the cerebral arterio-venous oxygen difference were determined on dogs breathing room air. In most experiments 2 cc of 1% neosynephrin solution were administered intramuscularly, portions of the 2 cc being injected at various sites. In a few experiments, however, 1-10,000 neosynephrin solution was injected intravenously and in these instances the amount of neosynephrin injected was much smaller than with intramuscular injection.

In Table I are presented the results of 2 out of 4 experiments on animals breathing reduced concentrations of oxygen. In all 4 experiments the blood became more alkaline as a result of the hyperpnea produced by breathing 11% oxygen and then less alkaline after injections of neosynephrin. Neosynephrin increased the concentrations of lactic acid and glucose in the blood. Blood pressure was

* We are grateful to Dr. Richard M. Johnson of the Frederick Stearns & Company for his generous supplies of Neosynephrin Hydrochloride.

† Aided by a grant from the Albion O. Bernstein Memorial Fund.

‡ We are grateful to Dr. E. Oppenheimer of the Ciba Pharmaceutical Products for his generous supplies of Dial Urethane Solution.

1 Himwich, H. E., Bowman, K. M., Daly, C., Fazekas, J. F., Wortis, J., and Goldfarb, W., *Am. J. Physiol.*, 1941, **132**, 640.

2 Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **51**, 523.

3 Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, 1923, **135**, 63.

4 Friedemann, T. E., Cotonio, M., and Shaffer, P. A., *J. Biol. Chem.*, 1927, **73**, 335.

5 Bock, A. V., Dill, D. B., Hurxthal, L. M., Lawrence, J. S., Coolidge, T. C., Dailey, M. E., and Henderson, L. J., *J. Biol. Chem.*, 1927, **73**, 749.

6 Dill, D. B., Edwards, H. T., Florkin, M., and Campbell, R. W., *J. Biol. Chem.*, 1932, **95**, 143.

TABLE I.
Effects of Injection of Neosynephrin on Oxygen Relationships of Dogs Respiring 11% Oxygen Under Dial.

			O_2 content, vol. %	O_2 capacity, vol. %	Hemoglobin satur., %	O_2 tension, mm Hg	pH	Sinus blood flow, cc/min	Blood pressure, mm Hg.	Glucose, mg %	Lactic acid, mg %
Dog No. 1	Room air	Art.	20.0	21.1	95	100	7.17	8.4	90	112	17
		Ven.	8.3		39	33	7.15				
		Diff.	11.7		56	67					
	11% O_2	Art.	11.5	22.3	52	34	7.28	9.0	105	117	18
		Ven.	2.8		13	16	7.20				
		Diff.	8.7		39	18					
	11% O_2 + Neosyn.	Art.	12.0	22.3	54	37	7.23	11.4	180	166	31
		Ven.	4.4		20	24	7.15				
		Diff.	7.6		34	13					
Dog No. 2	Room air	Art.	17.6	18.5	95	90	7.30	7.4	110	95	57
		Ven.	10.3		56	38	7.23				
		Diff.	7.3		39	52					
	11% O_2	Art.	14.6	18.4	82	53	7.35	8.2	120	170	63
		Ven.	5.1		29	22	7.26				
		Diff.	9.5		53	31					
	11% O_2 + Neosyn.	Art.	11.1	20.0	55	34	7.31	12.0	154	197	66
		Ven.	5.1		26	23	7.23				
		Diff.	6.0		29	11					

TABLE II.
Effect of Neosynephrin on Cerebral Arterio-Venous Oxygen Differences, Blood Sugar and Blood Pressure of Dogs Respiring Room Air.

	Neosynephrin		O_2 content, vol. %	Glucose, mg %	Blood pressure, mm Hg.
Dog No. 1	Before	Art.	22.4	100	125
		Ven.	11.3	93	
		Diff.	11.1	7	
	After	Art.	22.0	114	150
		Ven.	13.6	111	
		Diff.	8.4	3	
Dog No. 2	Before	Art.	23.8	107	100
		Ven.	13.4	103	
		Diff.	10.4	4	
	After	Art.	22.6	124	133
		Ven.	14.3	118	
		Diff.	8.3	6	

elevated following the injection of neosynephrin and accordingly blood flow through the brain accelerated.[7] As a result of the more rapid cerebral blood flow the cerebral arterio-venous oxygen difference was diminished and the difference in tensions of the oxygen of arterial and venous blood was also decreased. Table II reveals similar results in 2 of the 8 experiments made on animals breathing air. In each of these experiments the arterio-venous oxygen difference decreased after the injection of neosynephrin because the oxygen content of the venous blood rose. According to Courtice[8] the pressure of oxygen in the venous blood is representative of that in the brain. It is, therefore, suggested that the effect of neosynephrin to raise the oxygen of the venous blood may produce a similar change in the brain.

[7] Forbes, H. S., *Arch. Neurol. and Psychiat.*, 1940, **43**, 804.

[8] Courtice, F. C., *J. Physiol.*, 1941, **100**, 198.

14194 P

Circulatory Effects from Pentothal Sodium Administered Soon After Hemorrhage.*

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A death during sodium ethyl 1-methyl-butyl thiobarbituric acid (pentothal) intravenous anesthesia, given shortly after acute, inadequately treated hemorrhage prompted a study of its effects on circulation when given to animals during similar circumstances.

Method. Nineteen experiments were performed on 7 cats and 10 dogs. Arterial blood pressure determinations were recorded by cannulating a carotid or femoral artery during infiltration anesthesia. Control mean blood pressure readings averaged 127 mm Hg in both species. Hemorrhage was produced by direct arterial bleeding (averaging 6-8 minutes) until the mean arterial blood pressure level was reduced to an average of 70 mm Hg. Twenty-five to 30% of the estimated blood volume was withdrawn.

Freshly prepared 2% solutions of pentothal given intravenously at an average rate of 3.4 cc per minute were administered at a time averaging 24 minutes following bleeding. Subanesthetic amounts were used because in earlier experiments, not included in this study, the recommended anesthetic dose for normal dogs and cats when administered following hemorrhage caused prompt circulatory failure and death. All animals used had responded normally to the full anesthetic dose several days before being subjected to experiment. The usual dose of 25 mg/kg for dogs was reduced to an average of 11 mg/kg and cats were given 12 instead of the recommended 40 to 50 mg/kg. No preanesthetic medication was given.

An additional cat and 2 dogs were similarly prepared but received no pentothal. They were observed for 4 hours. After the initial fall following hemorrhage, no rapid fluctuations in blood pressure were observed for the control period.

Oxygen was administered by tracheotomy or endotracheal airway to 3 cats and 4 dogs preceding and during pentothal injection. Its use had no effect upon the results obtained.

Results. Following the administration of pentothal the animals exhibited 2 types of reaction. In all 7 cats and in 7 dogs the following sequence of events was noted. There was an initial rapid additional fall in blood pressure level to an average mean pressure of 29 mm Hg. This phase continued for 1 to 16 minutes. It was succeeded by a rise to an average of 65 mm Hg. in 1 to 12 minutes. Following this a secondary fall to the low level of 12 mm in 0.5 to 20 minutes occurred which caused the death of 4 cats and 3 dogs.

In 5 dogs, 3 exhibited an initial rapid fall in blood pressure to an average 50 mm Hg. which was succeeded by a phase during which blood pressure was elevated and maintained at an average of 105 mm Hg until the hypnotic effect wore off.

Two dogs with sustained blood pressure were given additional identical doses of pentothal when the clinical effect of the previous one had worn off. The reaction to each injection was identical to that preceding except that without warning one dog died following the third additional injection and the other after the fourth additional dose.

The pulse rate, increased after hemorrhage, slowed considerably in 13 of the animals, increased in 4, and was unaffected in 2 after pentothal was given. A decrease in pulse rate is not usually observed when pentothal is administered to animals with normal circulation.

Respirations were depressed after pentothal. They either became shallow, without increase in rate, or ceased completely.

Gross autopsies on the animals which succumbed revealed a markedly dilated heart due almost entirely to right-sided enlargement.

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The large veins were also greatly dilated.

Discussion. These few preliminary observations indicate that following hemorrhage the circulatory effects of pentothal sodium are unpredictable. In 14 of 19 experiments they were rapidly deleterious.

These observations on laboratory animals suggest that due consideration should be given the occurrence of recent hemorrhage when pentothal is to be used intravenously for anesthesia. There is a tendency from previous experimental work[1] to consider barbiturates desirable during traumatic operations. However, the impression that pentothal is safe and even beneficial in "shocked" cases[2,3,4] is not established and should be tempered.[5,6]

[1] Seeley, S. F., Essex, H. E., and Mann, F. C., *Ann. Surg.*, 1936, **104**, 332.

[2] Fordyce, C. Y., *Surg. Clin. No. Amer.*, 1942, **22**, 1483.

[3] Pender, J. W., and Lundy, J. S., *War Med.*, 1942, **2**, 193.

[4] Adams, R. C., and Gray, H. K., *Anesthesiology*, 1943, **4**, 70.

[5] Beecher, H. K., McCarrell, J. D., and Evans, E. I., *Ann. Surg.*, 1942, **116**, 658.

[6] Halford, F. J., *Anesthesiology*, 1943, **4**, 67.

14195

Studies on Muscle and Nerve in Biotin-Deficient Rats.*

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Several investigators have reported that rats reared on biotin-deficient diets develop a motor syndrome which is characterized by abnormality of locomotion, varying degrees of paralysis and spasticity. Nielsen and Elvehjem[1] have shown that this condition can be prevented and cured by crystalline biotin. Shaw and Phillips[2] reported that the skeletal muscles of biotin-deficient rats showed histologic evidence of atrophy but the spinal cord and peripheral nerves appeared quite normal. This report is concerned with a study of the functional state of muscles and nerves of biotin-deficient animals and includes observations on the rate of neuromuscular regeneration in such a deficiency.

Methods. Albino rats were reared from weaning on the basal egg-white ration of Nielsen and Elvehjem.[1] After symptoms characteristic of biotin deficiency appeared, studies were carried out on the gastrocnemius muscles and tibial nerves. In a number of animals the tibial nerve of one limb was crushed and the rate and extent of neuromuscular regeneration compared to that of control animals on an adequate diet. In the regeneration experiments the unoperated tissues of the contralateral limb served as controls. The strength of the muscles was determined by measuring the maximum isometric tension which developed in response to volleys of adequate stimuli applied directly to the muscle and to its motor nerve. The technics that were employed for denervation and strength measurements have been described in detail elsewhere.[3] In addition, observations were made concerning muscle and body weight ratios, creatine, chloride, and water concentration in the muscles.

Results. Because of an unpredictable survival time, the regeneration studies were conducted on animals which possessed the characteristic biotin deficiency symptoms but had not yet reached the terminal spastic condition. The muscles and nerves of such animals (Table I) were found to regenerate at rates compar-

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

[1] Nielsen, E., and Elvehjem, C. A., *J. Biol. Chem.*, 1942, **144**, 405.

[2] Shaw, J. H., and Phillips, P. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 406.

[3] Hines, H. M., Thomson, J. D., and Lazere, B., *Am. J. Physiol.*, 1942, **137**, 527.

TABLE I.
Average Values for Muscles of Control and Biotin-Deficient Rats.

Diet	No. rats	Time after lesion, days	Relative weight of denervated muscle*	Relative strength of denervated muscle* when activated through		Tension/g control muscle
				Nerve	Muscle	g
Control	14	21	68.3	40.1	51.6	1542
Biotin-defic.	8	21	71.4	39.4	42.7	1649
Control	14	28	78.9	65.9	68.8	1639
Biotin-defic.	5	28	85.9	58.8	73.6	1653

* Expressed in % of that in unoperated contralateral control.

able to those found for the tissues of control animals. If biotin is essential for neuromuscular regeneration, the requirements must be below that afforded by the prevailing dietary conditions. At the above mentioned stage of biotin deficiency, the strength per unit weight of the non-denervated gastrocnemii was not significantly different from that of control animals (Table I). Furthermore, the ratio of the muscle tension elicited through nerve stimulation to that resulting from direct muscle stimulation was not significantly altered, being 91.7% for the control animals and 93.4% for the biotin-deficient group. These deficient animals also exhibited normal creatine concentrations as well as normal values for the ratios of the gastrocnemius muscle weight to body weight.

Additional studies were made upon a second group of 18 rats which had reached the terminal stage of biotin deficiency characterized by a loss of body weight and an abnormal spastic gait. The creatine concentration (mg/100 g muscle) averaged 505 for the deficient and 452 for control animals of a comparable age; thus confirming the finding of Nielsen and Elvehjem[1] that there is a rise in the creatine concentration during the terminal stages of biotin deficiency. The chlorides averaged 1.36 mM/100 g wet muscle for the control group and 1.49 mM for the deficient animals, while the water content was normal. These findings indicate that the increased muscle creatine did not result from a decreased connective tissue phase. The muscles of this group showed a 24% reduction in strength per unit weight when compared to the muscles of control animals in good nutritional state. However, there was no significant deviation from normal in the ratio of the tension developed as a result of direct muscle stimulation to that developed upon stimulation of the motor nerve.

Our findings of normal muscle strength, creatine concentration, and neuromuscular relationships for the animals in the pre-spastic condition indicate that there are no functional muscle abnormalities at this stage of deficiency. With respect to the group of animals in the later deficiency states our observation of unimpaired neuromuscular relationships together with the absence of neural lesions as indicated by the studies of Shaw and Phillips[2] afford no explanation for the disturbance of locomotion seen in these rats. The fact that such animals are sensitive to handling suggests that the reflex effects of painful stimuli upon the motor behavior in this condition should be evaluated. Although the somewhat reduced muscle strength might conceivably indicate a specific effect of extreme biotin deficiency on the functional capacity of skeletal muscle, the loss in body weight and increased creatine concentration accompanied by a loss of muscle strength are data more suggestive of the conditions encountered during certain stages of non-specific inanition atrophy.

Summary. At no stage of biotin deficiency was the capacity of the motor nerve to elicit tension in its muscle found to be impaired. Only in the case of animals which were allowed to approach terminal states was there a reduction in the strength of the skeletal muscle. The rates of neuromuscular regeneration following denervation by nerve crush were not significantly different from those found for control animals on adequate diets.

14196

Effect of Atropine upon Atrophy and Neuromuscular Regeneration.*

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The following is a report concerning the effects of atropine upon denervated skeletal muscle and neuromuscular regeneration. Levine *et al.*[1] have reported that large doses of atropine decreased the rate of atrophy of denervated skeletal muscle of the rat. Fischer[2] found that large doses of atropine did not retard the diminution of power and birefringence in denervated gastrocnemius-solei of rats and pointed out that the retardation of atrophy was apparent rather than real inasmuch as the non-denervated contralateral control muscles lost appreciable amounts of weight during the experimental periods.

Methods. The studies were made on the gastrocnemius-soleus muscles and tibial nerves of 3- to 5-months-old rats. The experimental and control animals were matched as to age, sex, and stock. In some animals a section of the tibial nerve was removed in order to exclude reinnervation. In other animals the tibial nerve of one limb was crushed and allowed to regenerate. The muscles and nerve of the unoperated contralateral limb served as controls. The groups of treated animals received daily injections of atropine sulfate in doses of 10 to 15 mg per 100 g of body weight. Studies were made on the muscles at 2 or 3 weeks after denervation. These included measurements of the capacity of the muscles to develop isometric tension in response to stimuli applied directly to the muscles and to the nerves according to methods described in detail in a previous report.[3] Additional studies included determinations of muscle weight, creatine concentration and measurements of oxygen consumption by muscle strips in Barcroft-Warburg manometers.

Table I summarizes the results of the various experiments. Atropine was without significant effect upon the creatine concentration and the *in vitro* oxygen consumption of normal and denervated muscle. The daily administration of this drug for a period of 2 weeks had no effect upon the strength per weight unit of denervated and control muscle. The tension per g of the regenerating and control muscles of animals receiving atropine for 21 days was somewhat lower than in comparable muscles of non-treated control animals. The average amount of tension developed by non-denervated muscles per g of body weight was lower in atropine-treated animals than in the non-treated groups. The cause of the low muscle to body weight ratios in the non-denervated muscles of atropine-treated animals is not clear. Fischer[2] suggested that this was due to the effects of a cachectic inanition resulting from anorexia. In our experiments there was no correlation between the amounts of body weight loss and the decrease in the muscle-body weight ratios in the different animals. However, it was noted that the atropine-treated animals lost weight in the early days of treatment and tended to regain weight during the later days of treatment. It is probable that total body weight was regained relatively faster than muscle weight in the periods following inanition. However, a specific wasting effect of atropine on skeletal muscle may also be involved. When the extent of atrophy or amount of regeneration is estimated solely from the terminal differences in weights of denervated and non-denervated muscles it can be calculated that the muscles of atropine-treated animals exhibited a slower rate of denervation atrophy and faster regeneration than those of non-treated controls. The unreliability of employing such a calculation for estimating the rate of atrophy

* Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

1 Levine, R., Goodfriend, J., and Soskin, S., *Am. J. Physiol.*, 1942, **135**, 747.

2 Fischer, E., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 208.

3 Hines, H. M., Thomson, J. D., and Lazere, B., *Am. J. Physiol.*, 1942, **137**, 527.

TABLE I.

Average Values on the Gastrocnemius and Soleus Muscles of rats. D indicates denervated muscle and C signifies non-denervated contralateral control muscle. The *in vitro* O_2 consumption was measured on the solei. All other values refer to gastrocnemii.

Condition	No. of rats	Nerve lesion Type	Days	Body wt, g Initial	Final	Gms tension per g muscle D	C	Muscle wt* / Final body wt D	C	Muscle tension (g) / Final body wt (g) stimulated through Muscle D	C	Nerve D	C	Muscle creatine mg/100 g D	C	QO_2 D	C	% atrophy†
Control	13	cut	14	183	188	965	1857	.358	.588	3.34	10.62			344	453	2.7	2.5	39.1
Atropine	10	cut	14	186	181	957	1879	.342	.533	3.25	9.37			321	459	2.5	2.4	35.2
Control	7	crush	21	255	243	1269	1830	.378	.595	4.22	10.82	2.35	10.75	392	483			36.3
Atropine	5	crush	21	230	198	1078	1557	.354	.528	3.82	8.27	2.23	7.89	383	469			32.4

* × 100.

† Calculated from the differences in weight between denervated and non-denervated control muscle.

or degree of regeneration under conditions in which control muscle undergoes appreciable changes in weight during the period of experimentation is obvious.

Summary. The daily administration of large doses of atropine to rats failed to retard the rate of atrophy of denervated muscle and did not enhance the rate of neuromuscular regeneration. Atropine did not significantly affect the creatine concentration and *in vitro* oxygen utilization of denervated and control muscle. When expressed as a fraction of the final body weight the strength and weight of the control muscles of the atropine-treated rats were found to be inferior to those of untreated control muscles. The change in weight of the contralateral control muscle following atropine administration invalidates any estimation of the extent and rate of atrophy or regeneration based solely on the differences between the terminal weights of the control and experimental muscle.

14197

Reproduction of Bacteria from the Large Bodies of *Streptobacillus moniliformis*.*†

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In most strains of *Streptobacillus moniliformis* the bacteria develop by gradual swelling into large round forms. These forms transplanted under appropriate conditions germinate by the growth of small soft granules and diphtheroid-like forms and produce a pleuropneumonia-like, L1, colony.[1] Similar enlargement of bacteria and development to pleuropneumonia-like colonies was observed in the cultures of *B. funduliformis, B. coli,* and *B. influenzæ*.[1] In a culture of *B. coli*[2] and in the cultures of several strains of

* The expenses of this investigation have been defrayed in part by a grant from the Commonwealth Fund.

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1 Dienes, L., *T. Bact.*, 1942, **44**, 37.

2 Dienes, L., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 773.

3 Dienes, L., and Smith, W. E., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 297.

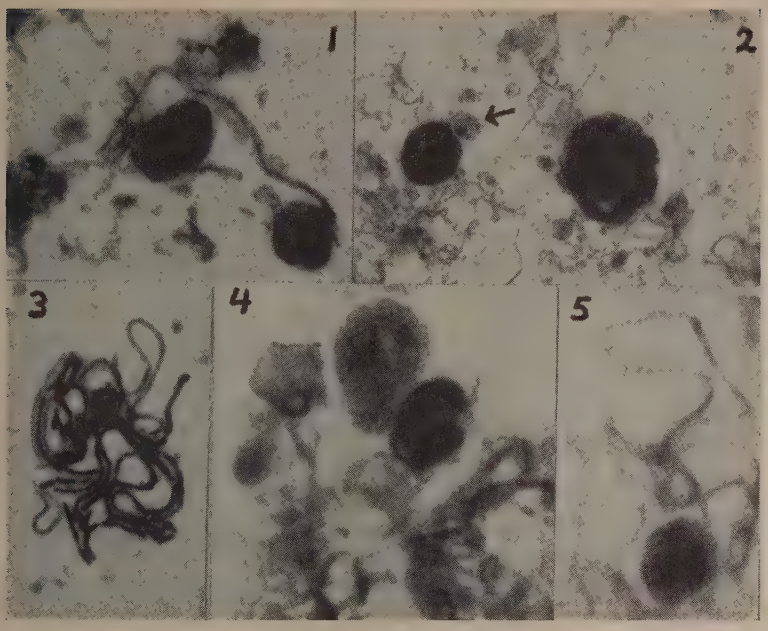

FIG. 1.

The photographs were taken from Giemsa stained impression preparations following Klieneberger's agar fixation technic.[4]

1. Represents the surface before incubation of an agar block just inoculated with a 48-hour culture. Bacterial filaments, large bodies, and debris resulting from the autolysis of the culture are visible in the photographs. Bacteria cannot be seen in the large bodies. × 2000.

2. Surface of the agar after 8 hours incubation as seen with moderate magnification. (× 1000.) Two round dense bacterial colonies are visible in the picture. The bacterial filaments of the inoculum show no growth. The round body marked with an arrow is full of tiny bacilli.

3. A young colony resulting from the growth of the bacterial filaments of the inoculum (10 hours incubation). (× 2000.)

4-5. Show large bodies packed with bacillary forms (high magnification × 3000). Note the smallness of the bacillary forms in the large body in photograph 5.

B. fundulifórmis[3] the large bodies went through another type of development and produced bacteria of the usual shape and size. A similar process was observed recently in a strain of *Streptobacillus monilifórmis*.

The strain which showed this phenomenon was received from Doctor Heilman. It was preserved without being transplanted in CO_2 refrigeration. The strain grew in small colonies which after 24 hours consisted of intertwined filaments with relatively few large bodies. After 48 hours the colonies consisted mostly of very large flat bodies (up to 20 microns in diameter) filled with vacuoles of various size. The bacterial filaments in these colonies were to a large extent autolyzed and stained only faintly. L1 colonies did not develop beneath the bacterial colonies but they developed in moderate numbers among the bacterial colonies in transplants. The bacterial growth in young cultures often was first noticeable in the form of very tight round colonies in contrast to the usual loose colonies of the streptobacillus. This observation suggested that the bacterial growth started from the large bodies.

To examine this supposition, agar blocks inoculated with 24- and 48-hour-old cultures were incubated on coverslips. The blocks after varying lengths of incubation were fixed

with Bouin solution.[4] The coverslips were stained with Giemsa and differentiated with tapwater and ascitic fluid. The blocks were incubated between 25-30°C. Bacterial forms were never visible in the large bodies in the original cultures or immediately after transplantation. After a few hours of incubation, however, many large bodies appeared tightly filled with bacteria and their growth produced the round dense colonies. Bacteria developed more often in the large bodies transplanted from 48-hour-old cultures than from 24-hour cultures. The appearance of the culture and the large bodies filled with bacteria are illustrated in the photographs (Fig. 1).

The bacteria according to all probability were produced inside the large bodies by transformation of the contents of the large bodies. They did not grow in from the outside. We mentioned above that bacteria were never visible in the large bodies in the original cultures. They appeared after as short incubation as 1½ hours and were quite numerous after 5 hours. The bacterial multiplication did not start during this period. The large bodies were either completely filled with bacterial forms or contained none. One never saw only a few bacteria or bacteria in vacuoles as occasionally occurs in bacterial contamination of cultures of L1. The bacteria inside the large bodies are much smaller and thinner than the bacteria which later start to grow outside of them. Bacteria are never visible in the autolyzed large bodies which do not stain well. They are always in those which are deeply stained and well maintained. The growth of bacteria from the content of large bodies was observed in 2 other bacterial species, which makes it easier to accept that a similar process occurs in *Streptobacillus moniliformis*.

Apparently in all species the large bodies possess a double potentiality to develop either into regular bacteria or into pleuropneumonia-like (L) forms. The L forms under appropriate conditions return to regular bacterial morphology. They might represent intermediate forms in the development of the bacteria inside the large bodies. These forms in certain cases progress immediately into regular bacterial forms; while in others they are able to grow in this intermediate form.

[4] Klieneberger, E., and Smiles, T., *J. Hygiene*, 1942, **42**, 110.

14198

A Toxic Factor Associated with the Agent of Lymphogranuloma Venereum.

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The production of a toxic factor during the development of the agent of lymphogranuloma venereum has been suspected for the following reasons: (A) The disease can occur in man in severe generalized form[1] with marked headache, chills, and other symptoms suggesting toxemia. (B) The cause of death of the chick embryo after yolk-sac infection is most readily explicable as due to a toxin, since the agent does not invade the embryo to any great extent[2] and disturbance of nutrition would appear unimportant since weight of embryos dead of the infection does not differ from that of uninfected embryos of the same age. (C) In mice, convulsive deaths occurring some 30 hours after intracerebral inoculation suggest toxemia and are not wholly accounted for by the lesions formed in the meninges. Bearing on this problem Gildermeister and Haagen

[1] Harrop, G. A., Rake, G., and Shaffer, M. F., *Trans. Am. Clin. and Clim. Assn.*, 1941, **56**, 154; *Trans. Assn. Am. Phys.*, 1941, **56**, 101.

[2] Rake, G., and Jones, H. P., *J. Exp. Med.*, 1942, **75**, 323.

TABLE I.
Toxic Factor in Yolk-Sac of Moribund Embryo.

Dilution of "Toxin"					
				*Sediment	4, <15, <15, <15
1/5	†5,	5,	6,	6, 7	
1/10	<18,	<18,	<18,	<18, 23	
				*Supernate	S, S, S, S
1/20	<18,	<18,	21,	24, S	

Original (1/5) dilution in infected yolk. Further dilutions in normal yolk. 12 g mice used. All inoculations 0.5 ml intravenously.

* Original dilution centrifuged at 18,000 r.p.m. in cold for 1 hr. Sediment resuspended to original volume.

† 5 = died in 5 hr. S = Survived.

have demonstrated a "toxin" from rickettsia grown in the yolk-sac of the developing embryo.[3]

A toxic product has been demonstrated in the heavily infected yolk-sac of moribund embryos. Little is present in less heavily infected yolk-sacs, *i.e.*, those from embryos which would not die for another 12 or 24 hours, and it disappears after death if the eggs remain in the incubator or more slowly if at room temperature. Yolk-sacs are shaken in their own yolk and fluids to make a 1/5 or 1/10 suspension and further dilutions are prepared in broth, normal yolk, or normal serum (human, rabbit, or calf). Even under favorable conditions the toxic factor is not present in large amounts, and it has not been possible to demonstrate it in dilutions of infected yolk-sac higher than 1/40 or of infected yolk higher than 1/2 (Table I).

The lethal action can be demonstrated in 10 to 20 g mice following intravenous inoculation of 0.5 ml volumes. Death also occurs within 17 hours after intraperitoneal inoculation of 2 ml of a 1/5 suspension. After intravenous inoculation mice may die in 4 hours and most fatalities occur within 24 hours. Convulsions may precede death particularly when this is rapid. That death is due to a toxic product and not to infection is shown by the rapidity of death in many mice (mice infected intracerebrally with the agent of lymphogranuloma venereum, the most rapid method, very rarely die before 30 hours, and usually take 36 to 48 hours); by the fact that the mice either die acutely or become perfectly well while infected animals which survive show all degrees of chronic disease; and by the fact that microscopic examination of the organs reveals no evidences of infection. The tissues do, however, show the results of the "toxin." Focal lesions are found in the brain; small areas of edema and hemorrhage occur in the lung; and the kidneys on occasion show fibrin thrombi in the glomeruli and destruction of the tubular epithelium. Most constant and important, however, are numerous foci of necrosis in the liver which recall those seen in livers of mice dying from psittacosis.[4] It may be stated that it has recently proved possible to demonstrate a similar toxic factor connected with the agent of psittacosis. This "toxin" is more lethal, or is present in larger amounts, than is that of the agent of lymphogranuloma venereum.[5]

The toxic factor seems to be associated with the bodies of the agent. Thus when infected yolk-sacs shaken in infected yolk are first centrifuged at 1500 r.p.m. and the supernate from this then recentrifuged at 18,000 r.p.m. for 1 hour in the cold, the "toxin" is found in the resuspended final sediment of elementary bodies and not in the supernate from which 99% of the agent has been removed[6] (Table I). Similarly it is lacking in any filtrates in which the agent is held back by the filter. Sediment prepared in a similar way from normal yolk-sacs gives no evidence of toxicity.

Antibodies are produced against the toxic factor. Thus serum from rabbits immunized with suspensions of the agent will prevent the

[3] Gildermeister, E., and Haagen, E., *Deut. Med. Woch.*, 1940, **66**, 878.

[4] Rivers, T. M., Berry, G. P., and Rhoads, C. P., *J. Am. Med. Assn.*, 1930, **95**, 579.

[5] Jones, H., and Rake, G., to be published.

[6] Rake, G., McKee, C. M., and Shaffer, M. F., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 332.

TABLE II.
Resistance of Mice Recovered from Sublethal Dose of Toxic Factor.

Mice	Dilution of "toxin"					
Controls	1/10	<17,	<17,	18,	19,	<40
	1/20	22,	S,	S,	S,	S
	1/40	S,	S,	S,	S,	S
Recovered from sublethal dose 10 days before	1/10	<16,	23,	S,	S,	S
		S,	S,	S,	S	

All inoculations 0.5 ml intravenously.
All mice weighed 15-16 g.

lethal action of the "toxin" when mixed with it and the mixtures allowed to stand for 1 to 2 hours at room temperature before inoculation, or when injected intravenously a few minutes preceding the dose of toxin. Moreover, mice which have recovered from one sublethal dose of the toxic factor received 10 to 14 days before are more resistant to reinoculation than are control animals. (Table II.)

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